The Regenerative Role of Retinoids in Emphysematous Postnatal Mouse Lungs

Annan, Adelaide

Awarding institution:
King's College London

Title: The Regenerative Role of Retinoids in Emphysematous Postnatal Mouse Lungs

Author: Adelaide Annan

The Regenerative Role of Retinoids in Emphysematous Postnatal Mouse Lungs

Thesis submitted for degree of

DOCTOR OF PHILOSOPHY

By

Adelaide A Annan

Developmental Biology

MRC Centre, Developmental and Neurobiology

King's College London

April 2014

Supervisors: Patrick Doherty and Guy Tear

ABSTRACT

Debilitating lung disorders such as chronic pulmonary disease (COPD) and bronchopulmonary dysplasia (BPD) are presently incurable. The World Health Organization (WHO) predicts that by 2030, COPD will rise to become the third leading cause of death. Much of the increase in COPD is associated with the increase in tobacco use and the exposure to smoke combustion from fuel. COPD includes chronic bronchitis and emphysema. It is caused not only by inhalation of polluted air, but also by infections as well as genetic predispositions.

To protect the respiratory airways, goblet cells in the bronchiole epithelium produce and secrete a viscous substance known as mucus along with enzymes to breakdown and remove inhaled toxins. Repeated and prolonged exposure to these toxins cause an overproduction of both mucus and enzyme secretion to become uncontrollable. As a result, the airway epithelium becomes scarred and fibrotic. These enzymes breakdown the delicate alveolar cell walls creating enlarged alveoli space. Elastic fibers in these cell walls are also destroyed resulting in a loss of elastic recoil narrowing the airways thus obstructing airflow. It becomes difficult to obtain enough oxygen into the blood and to remove excess carbon dioxide. These changes lead to a shortness of breath and other symptoms.

Unfortunately, the symptoms of COPD cannot be eliminated with current treatment available and the condition inevitably worsens over time. Treatment available might not eliminate symptoms, but they can sometimes slow the progression of the disease.

Transplantation and oxygen therapy are two of the common forms of treatment. The problem with these therapies is that it requires the patient to be relatively healthy, so therefore assessable to few patients. One possible way to treat COPD would be to somehow induce regeneration in these lungs or to impart, self-heal. Unfortunately, adult lung tissue seems incapable of spontaneous repair therefore understanding how to activate repair mechanism would greatly improve the prospects of effective treatments and the prognosis for COPD patients.

Previous studies including the use of experimental adult rat model of emphysema has suggested one way to induce lung regeneration is via the endogenous metabolite of vitamin A, retinoic acid (RA). In the current study, we have described a mouse model of disrupted alveolar development using a dose-dependent glucocorticoid steroid, dexamethasone administered postnatally to create serve loss of alveolar surface area. When RA is induced to these animals as adults the lung architecture is restored to normal. This remarkable effect may be because RA is involved in alveolar development.

We also provide evidence that RA and its agonists are required for the ongoing maintenance of alveolar structure and function because rats deprived of dietary retinol lose alveoli and show pathological features of emphysema. Alveolar regeneration with RA and its agonists may therefore be an important part of a novel therapeutic approach for the treatment of respiratory diseases characterized by reduced gas-exchanging surface areas such as BPD and emphysema.

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Professor Patrick Doherty. He gave me the encouragement that I needed to complete this thesis. There were times where I really struggled to believe that I could complete this thesis and had to overcome so many obstacles, both professionally and personally, but he helped me stay on course. He is not only supportive and approachable, but has never made me feel silly for asking questions that you feel you should know the answer to.

Next I would like to thank Dr Åsa Gilthorpe, my postdoc in the Maden lab. She made science fun for me. She created a good work ethics and encouraged me to work the same. She was supportive and always happy to help me and willing to teach me new techniques and I will always be grateful for that. She is not only a good colleague, but a great friend.

I have known Brenda Williams and Carl (uncle) Hobbs all my scientific life. I consider them my family. They have always been there for me and always encouraged me not to listen to people who said I should give up or questioned me ever doing a PhD. I've known Carl since my days at St Thomas' Hospital and gave my mock exam for my state registration to become a state registered histologist. I thought it was really bad, he said I did really well! Brenda has always been on the other end of line if I needed to let off some steam, never judging me and always giving great advice. I had an awful lot of laughter with these two people.

I’d also like to thank my current group, Salivary Research and especially to my current boss Professor Gordon Proctor. He has been incredibly supportive, especially as my PhD isn’t even related to my current job. My colleagues in Salivary who have had to put up with my mood swings along with stress (that wasn’t fun)! Special thanks to my two best friends in Salivary Research, Drs Hannah Gibbins and Tom Richards and Mrs. Lauren Appleyard. I have loved every minute working with them. Talking to Hannah and Tom about writing up their thesis and seeing Hannah go through her PhD has only encouraged me further. They have been absolute rocks; thanks guys. A special thanks to Hina Gosrani, who volunteered to read some of my chapters and has also become a close friend.

Finally a massive thanks to my family and friends. It has at sometimes been incredibly tough for me, but having great people around me has made it all worthwhile. It is a shame my mum isn’t here to see this, but I hope she is looking down on me proud. Thank you all.

CONTENTS PAGE

List of Acronyms……..18

ABPAS: Alcian Blue Periodic Acid Schiff's Reagent

ATI: Alveolar Type I cells

ATII: Alveolar Type II cells

BAL: Broncho-Alveolar Lavage

BADJ: Bronchiolar-Alveolar Duct Junction

BPD: Bronchopulmonary dysplasia

BSA: Bovine Serum Albumin

CBB: Coomassie Brilliant Blue

CC10: Clara Cell Secretory protein10kDa

CF: Cystic Fibrosis

Cld: Claudin

COPD: Chronic Obstructive Pulmonary Disease

DAB: 3, 3'-Diaminobenzidine

Dex: Dexamethasone

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial Mesenchymal Transition

HRP: Horseradish Peroxidase

IF: Immunofluorescence

IHC: Immunohistochemistry

IL: Interleukin

Lm: Alveolar Space Intercept

μm: Microns

MMP: Matrix Metalloprotease

MUC: Mucin

Nkx2.1: Nk2 homeobox (also known as Thyroid Transcription Factor-1)

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

RA: Retinoic Acid

RAR: Retinoic Acid Receptor

RARα: Retinoic Acid Receptor Agonist Alpha

RARβ: Retinoic Acid Receptor Agonist Beta

RARγ: Retinoic Acid Receptor Agonist Gamma

ROS: Reactive Oxygen Species

RXR: Retinoid X Receptor

SDS-PAGE: Sodium Dodecyl Sulphate- Polyacrylamide

SP-C: Surfactant Protein-C

TBS: Tris Buffered Saline

TER: Transepithelial Resistance

TJ: Tight Junction

tRA: All-trans-RA

CHAPTER 1

INTRODUCTION

1.1. LUNG DEVELOPMENT: The lung is composed of branched tubes that that conduct air to a complex gas-exchange system. The gas-exchange surface is composed of sacs called alveoli, which are lined by a series of blood capillaries (Burri 2006). The development of the lung involves complex mesenchymal-epithelial interactions (Kumar, Lakshminrusimha et al. 2005, Tebockhorst, Lee et al. 2007). Based on histological features, the development of the lung can be classified into different stages (Fig 1.1). The embryonic stage; characterised by the formation of the lung bud as a simple budding of the epithelium from the ventral diverticulm of the foregut into the surrounding mesenchyme. The resulting bud undergoes rapid dichotomous branching via a complex epithelium-mesenchyme interaction. This is the pseudoglandular stage, characterised by the formation of the conducting airway tree down to the terminal bronchioles (Cardoso and Williams 2001, Cardoso and Lu 2006). Next is the canalicular stage, which shows the expansion of the airway tree and extensive organ growth along with differentiation of the airspace epithelium cell types. This stage is also marked by a vast increase in the number of capillaries in the primitive mesenchyme (Burri 2006). The main gas-exchange surface is formed in the next developing periods, the saccular and alveolar stages.

Transformation of the immature saccular lung which has a limited gas-exchange area, to the mature lung with a much larger internal surface area, entails the thinning of alveolar walls; growth of a capillary network and extensive subdivision of gas-exchange units. This period is the marked by interstitial fibroblast proliferation while epithelial cells flatten and decrease in numbers, resulting in a net thinning of distal airspace walls (Kresch, Christian et al. 1998). Concurrently, the alveolar capillary network becomes more complex. Alveolar septation begins as a secondary crest that

extends from the primary alveolar walls (Warburton and Lee 1999, Massaro and Massaro 2001, Burri 2006, Kim and Vu 2006). These crests or septa develop through deposition of new basement membranes, outgrowth of epithelial cells and myofibroblasts at the tips of septa and elastin deposition (McGowan and Torday 1997). Septation is developmentally regulated, occurring primarily from postnatal day 4 through to day 14 in rats and mice and during the last month of gestation and the first postnatal years in humans (Amy, Bowes et al. 1977, Burri 2006).

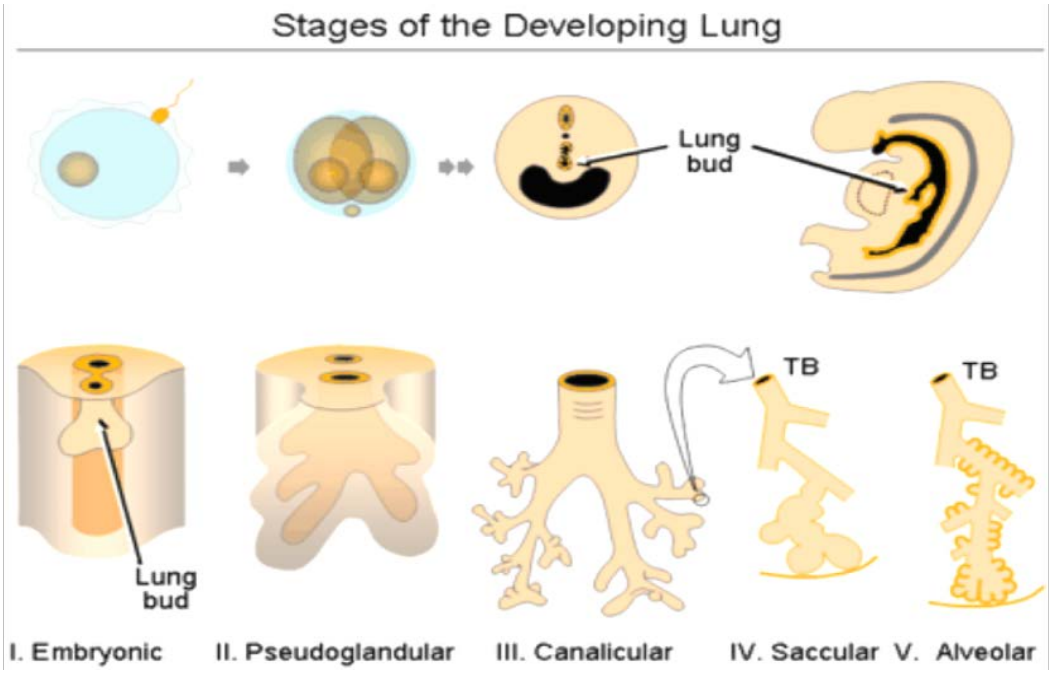

Fig 1.1: Stages of the developing mammalian lung. The formation of the lung undergoes five distinct stages; each with characteristic developmental features. (*Oxford Journal Vol 3 Suppl2*

Little is known about signals for the selection of specific septation initiation sites over others. A number of theories have been put forward to explain the crucial steps. Various stimuli, including genetic factors, oxygen tension, nutrition and hormones have been shown to modulate distal growth of the lung (Jobe and Bancalari 2001, Jobe and Ikegami 2001). These stimuli exert their effects via interactions with specific developmentally regulated molecular pathways that have been evolutionarily conserved (Burri 2006).

Many investigators agree that the initial deposition of elastin at specific sites in the walls of the developing saccules marks the onset of septa formation (Fig 1.2) (Li, Sorensen et al. 1999). Elastin gene expression peaks during alveolarization when elastin fibres localise to the tips of alveoli secondary crests, forming rings that surround the alveolar entrances when first expressed near the airway branching points during the pseudoglandular stage (Wendel, Taylor et al. 2000). With the alveolar walls, elastin fibres deposit into bundles. As elastic fibres are the primary tissue components in which mechanical stretch is stored and released in the septum, these fibres and elastin-expressing cells (myofibroblasts and fibroblasts) fundamentally determine septal mechanical properties and strain-related signal transduction. Mechanical stress provides signals for orientation and differentiation of alveolar myofibroblasts as well as directional organisation of the complex elastic fibre network (McGowan 1992, McGowan and Torday 1997).

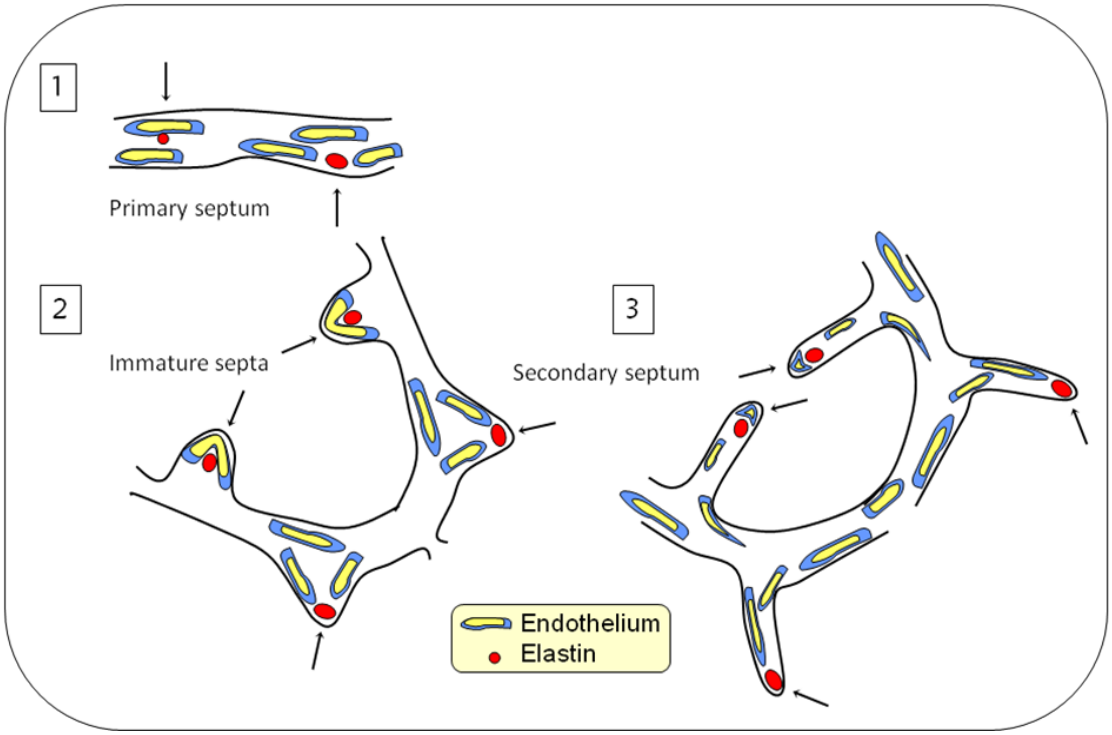

Fig 1.2: Schematic diagram illustrating the principle of the formation (alveolarization). Secondary septa (3) i.e. the future interalveolar walls, emerge from the primary septa (1) by the folding of one of the two capillary layers (2). Elastic tissue (red dots), play an important role in this process. Black lines depict each structural stage of alveolarization.

During the transition from the saccular to the alveolar stage, the walls of the terminal respiratory units become thinner by apoptosis and a single layer of capillaries replaces the double-capillary network (Fig 1.3) found in rudimentary alveolar walls (Kresch, Christian et al. 1998). For the septum to achieve its final natural morphology and role two pre-conditions are critical, namely the presence of a mature microvasculature and the thinning of the septal mesenchyme, which provides the shortest distance for gas-exchange.

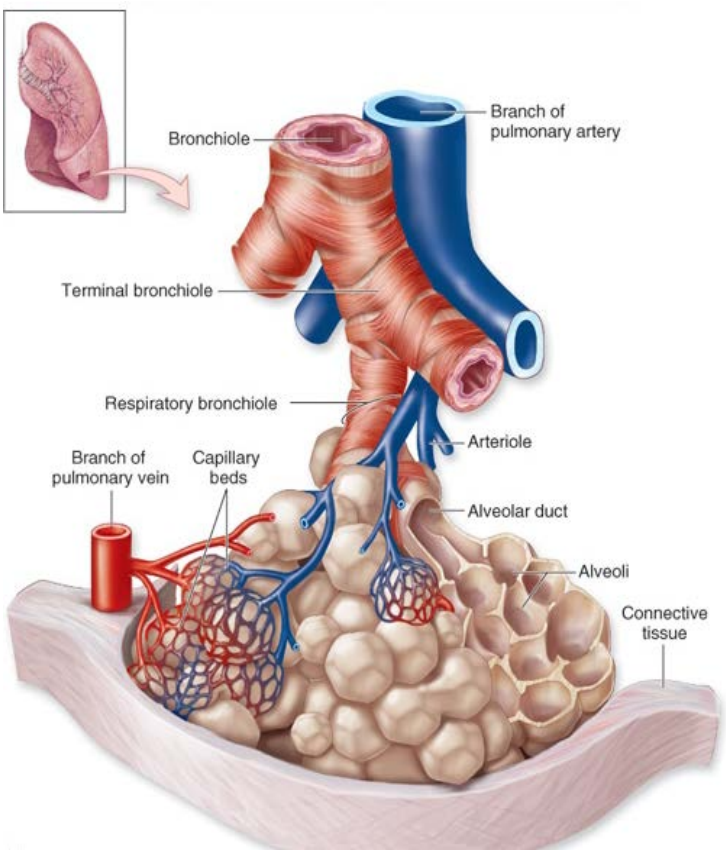

Fig 1.3: Schematic diagram of extensive capillary network of the alveolar epithelium. Formed lungs contain a large network of branching pulmonary veins and capillary buds, along with bronchioles and alveoli (honeycomb structure).

Vascular development occurs via two processes: (1) vasculogenesis, the development of blood vessels from the differentiation of angioblasts in the mesoderm and (2) angiogenesis, which is classically described as sprouting of blood vessels from existing vessels, but can also occur by “intussusception” i.e. the formation and growth of a transcapillary tissue pillar that eventually divides an existing capillary segment into two parts (Burri and Moschopulos 1992, Burri and Djonov 2002, Djonov, Kurz et al. 2002, Parera, van Dooren et al. 2005, Makanya, Hlushchuk et al. 2007). The bronchial vessels develop with preacinar airways; development is complete by 16 weeks gestation with further growth in size to match

lung growth. Bronchial vessels are generally not found in the peripheral acinar region and thus do not normally participate in alveolar gas exchange. Pre-acinar pulmonary arteries, supplied by the right heart, grow simultaneously with the airways into the intra-acinar region and fuse with the peripheral microvasculture that has arisen from the mesenchyme by vasculogenesis (Hislop 2002, Hislop 2005). It is not yet fully understood how capillary invasion and alveolar septation interact with each other. The importance of vascular supply to alveolarization was demonstrated by studies using antiangiogenic agents, e.g. inhibitors of the vascular endothelial growth factor (VEGF) receptor (Tsao, Li et al. 2004, Kunig, Balasubramaniam et al. 2005).

Thinning of the mesenchymal tissue involves apoptosis. Postnatally, there is a substantial reduction in the number of interstitial myofibroblasts resulting from increased apoptosis during alveolarization (Kresch, Christian et al. 1998).

1.2: CELLULAR COMPONENTS OF THE LUNG: The structure of the airway epithelium is pseudostratified in the large airways and becomes columnar and cuboidal in the smaller airways. There are numerous cells types in the respiratory system, but the main cellular types fall into the following category: ciliated, columnar, undifferentiated, secretory and basal cell (progenitor cells of the airway epithelium under certain conditions). In the large airways, cell types are ciliated, undifferentiated columnar, secretory and basal cells. Less common cell types in the large airway are cells such as cartilage, neuroendocrine cells and mucus glands. In the small airways, the cell types are similar to cells located in the large airways, but

are more ciliated and secretory cells shift to Clara cell types. The airway epithelium merges with alveolar epithelium type I and II (ATI and ATII).

DIVERSE EPITHELIAL CELLS IN THE MATURE LUNG: Maturation of the epithelium during development starts in the proximal airways and progresses distally into the intrapulmonary airways (Ayers and Jeffery 1988). Epithelial cell lineages are arranged in a distinct proximal-distal spatial pattern in the airways and become morphologically apparent during the pseudoglandular stage(Ten Have-Opbroek 1981). At least 11 different epithelial cell types have been described in the conducting and respiratory regions of the lung (Ayers and Jeffery 1988). The lineage relationship between the different cell types has not been delineated and the existence and identity of progenitor cells, which may play a role in lung injury and repair, are currently under study. There is some evidence from cell kinetic studies to suggest that basal cells, Clara cells and ATII cells are the primary progenitor cells from the pulmonary epithelium (Adamson and Bowden 1974, Evans, Cabral et al. 1975, Evans, Cabral-Anderson et al. 1978). Lineage studies have not addressed the relationship of pulmonary neuroendocrine cells to the putative stem cells (ATII cells and Clara cells) although neuroendocrine cells differentiate morphologically before any other epithelial cell type (Cutz 1982).

The mature mammalian lung is remarkably non-proliferative with the slow rates of mitosis and prolonged survival of resident cells. Nevertheless, after infection, resection, inflammation or toxicant exposure, the lung is capable of the rapid and extensive proliferation of various epithelial cell types. In the alveolar region, ATII

epithelial cells proliferate and differentiate rapidly into ATI cells as well as replace injured epithelial cells after exposure to various pathogens and toxicants (Fig 1.4). In conducting airways numerous cell types, including subsets of nonciliated columnar cells and basal cells, can proliferate. These progenitor cells or their progeny differentiate into other respiratory epithelial cell types, including Clara cells, goblet cells and ciliated cells. The respiratory epithelium is capable of rapid squamous metaplasia, proliferation and migration to replace the injured epithelial surface (Van Winkle, Johnson et al. 1999, Borthwick, Shahbazian et al. 2001, Hong, Reynolds et al. 2004, Rawlins, Ostrowski et al. 2007, Cardoso and Whitsett 2008).

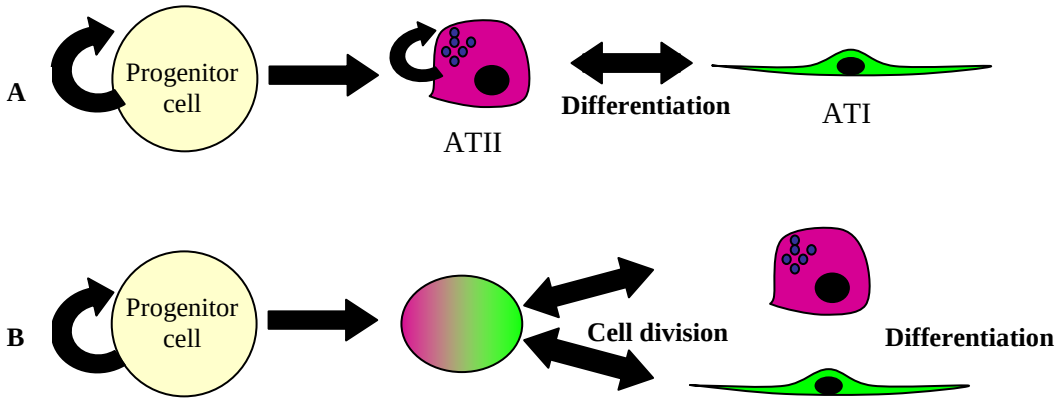

Fig 1.4: Cellular mechanism of the regeneration of ATII cells differentiating into ATI cells (A) and proliferating into more ATII cells (B).

The onset of cellular differentiation is signalled by the expression of differentiated gene products. Lung-specific gene products include the surfactant proteins; surfactant proteins A, B, C and D:- SP-A, SP-B, SP-C and SP-D and Clara cell secretory protein (CCSP) (Singh, Singh et al. 1988, Weaver and Whitsett 1991, Hackett, Shimizu et al. 1992, Wert, Glasser et al. 1993). The surfactant-associated proteins that are expressed primarily in ATII epithelial cells although SP-A and SP-B are also detected in subsets of nonciliated epithelial (Clara) cells of the conducting airways and tracheobronchial glands (Auten, Watkins et al. 1990, Phelps and Floros 1991, Weaver and Whitsett 1991, Khoor, Gray et al. 1993, Wert, Glasser et al. 1993, Khoor, Stahlman et al. 1994, Kuroki and Voelker 1994). Expression of these genes is extinguished when ATII cells undergo terminal differentiation to ATI cells that constitute most of the gas exchange surface of the alveolus. For the most part, CCSP is a marker for the proximal Clara cells of the bronchiolar epithelium (Singh, Singh et al. 1988, Hackett, Shimizu et al. 1992).

Early on embryonic development, undifferentiated epithelium co-express several lineage markers including SP-A and CCSP (Wuenschell,Sunday et al.1996). During the pseudoglandular stage, pulmonary epithelial cell lineages become restricted to proximal and distal regions of the airways. Interestingly, after bleomycin-induced injury in the adult lung, there is the appearance of co-expression of these lineage-specific markers, suggesting that a progenitor-type cell may be re-enlisted during epithelial repair (Daly, Baecher-Allan et al. 1998).

Within a restricted time frame of development, distal lung mesenchyme can reprogram rat tracheal epithelium to express tracheal epithelium to express ATII cell

differentiation and conversely, tracheal mesenchyme can induce distal lung epithelium to express tracheal cytodifferentiation (Shannon 1994, Shannon, Pan et al. 1998). These findings underscore the importance of epithelial-mesenchymal interactions in coordinating the precise temporo-spatial pattern of lung development.

TRANSCRIPTIONAL ELEMENTS: Analysis of transcriptional elements in surfactant protein genes and Clara cell protein has provided an understanding of the shared mechanisms of gene regulation and expression in respiratory epithelial cells. The homeodomain protein Nkx2.1 plays an essential role in several phases of lung development, including epithelial cell lineage determination (Guazzi, Lonigro et al. 1994, Minoo, Hamdan et al. 1995, Minoo, Su et al. 1999). Nkx2.1 is the earliest marker of the developing respiratory epithelium, with an onset of expression at the time of lung budding formation from the foregut endoderm (Lazzaro, Price et al. 1991). Distribution of Nkx2.1 expression in the foetal and mature lung includes respiratory epithelial cells of the trachea, bronchi and developing respiratory tubules (foetal lungs) (Lazzaro, Price et al. 1991, Ikeda, Clark et al. 1995). Expression in foetal lungs is most prominent in the distal alveolar cells, whereas in the mature lungs, the numbers in both alveolar and bronchi are almost equal.

Regulatory regions of all the surfactant protein genes are controlled by Nkx2.1 (Bohinski, Di Lauro et al. 1994, Maeda, Dave et al. 2007). Numerous experiments with null mutation of Nkx2.1 in mice have shown a failure to form bronchiolar and alveolar structures distal to the lobar bronchi and that pulmonary-specific gene expression including SP-B, SP-C and CCSP are turned off within transgenic lungs,

which do contain ciliated and mucus-secreting cells (Minoo, Su et al. 1999). Thus Nkx2.1 is considered by authors as a “master gene” that has the ability to induce and maintain cell lineages.

Numerous experiments support the concept that discrete anatomic regions harbour progenitor cells that are relatively protected from injury (Engelhardt 2001, Kim and Vu 2006, Cardoso and Whitsett 2008). Progenitor cells in these regions have unique capacities for both self-renewal and capability of proliferation and migration to repair injured respiratory epithelium. For example; basal cells, cells lining the neck of the tracheal-bronchial glands, toxic-resistant nonciliated cells (Clara cells) residing near neuroepithelial bodies (NEBs) and cells within the bronchoalveolar duct junction have been proposed to be uniquely capable of serving as progenitor cells after extensive epithelial cell injury (Hong, Reynolds et al. 2004, Cardoso and Whitsett 2008).

1.3: DISEASES OF THE RESPIRATORY SYSTEM: CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AND MANAGEMENT.

RESPIRATORY DISEASES: Respiratory diseases can be classified under the following categories; by the way the organ of the tissue is involved, by the pattern of associated signs and symptoms or by the aetiology of the disease. The major types of disease include:

1. **Inflammatory lung disease:** This is characterised by high neutrophil cell count which can be found in diseases including asthma, cystic fibrosis, emphysema, chronic obstructive pulmonary disorder or acute distress syndrome.
2. **Obstructive lung diseases:** Obstructive lung diseases are diseases of the lung where the airways (i.e. bronchi, bronchioles, alveoli) become reduced in volume or where free flow of gas is impeded, making it more difficult to move air in and out of the lung.
3. **Chronic Obstructive Pulmonary Disease (COPD):** This includes emphysema as an example of obstructive lung disease. This is where alveoli rupture causing the air to be retained in the lungs and limiting the availability of space during inhalation. Asthma also falls into this category.
4. **Bronchopulmonary Dysplasia (BPD):** BPD is a chronic lung disease, commonly associated with premature infants who are in need of mechanical ventilation and oxygen therapy, often from the result of a restricted dietary intake of vitamin A. It may also occur in premature infants who have had few signs of initial lung disease. Infants at risk of developing BPD are born below 28 weeks gestation, at a stage where parallel processes of alveolarization of the distal lung saccules and development of the capillary bed are just starting to take form.
5. **Lung Hypoplasia:** The aetiology of this disorder includes prolonged rupture of membranes in the lungs, foetal renal dysplasia and obstruction and foetal neuromuscular diseases. Lung Hypoplasia also occurs in association with congenital diaphragmatic hernia and congenital cystic lung lesions such as cystic adenomatoid malformation. High risk congenital diaphragmatic hernia survivors, who were mechanically ventilated, show an incidence of more than 30% in BPD (Bos, Hussain

et al. 1993). Pulmonary Hypoplasia or aplasia is part of the spectrum of malformations characterised by incomplete development of the lung (deMello 2004). The severity of the lesion depends on the timing of the insult in relation to the stage of lung development and the presence of other anatomic anomalies. The Hypoplastic lung consists of a carina (a malformed bronchial stump) and absent or poorly differentiated distal lung tissue.

1.4: ANIMAL MODELS OF THE CHRONIC OBSTRUCTIVE PULMONARY DISEASE: COPD

COPD: Chronic Obstructive Pulmonary Disease (COPD) is the fifth leading cause of death in the UK. It is also a global respiratory problem, reaching almost epidemic proportions in the developing world. Molecular and cellular research into the mechanics of COPD and the prevention of this disease is still in its infancy and despite the seriousness of this disease, medically it receives very little attention when compared with other conditions such as heart disease and cancer.

COPD is a disease characterised by airflow limitation that is not fully reversible, and is usually both progressive and associated with an abnormal inflammatory response of the lungs to noxious particles or gases. The result is an accelerated decline in lung function, expressed as the forced expiratory volume in one second (FEV1) and is ratio to the forced vital capacity (FVC) (Pauwels 2001, Brusselle, Bracke et al. 2006). Cigarette smoke is by far the most common cause of COPD. Passive exposure to cigarette smoke (i.e. environmental tobacco smoke) may also contribute

to respiratory symptoms and COPD(Brusselle, Bracke et al. 2006). COPD does not only derive from tobacco smoke and environmental exposure, but genetic factors. Thus, the study of gene-environmental interactions is of one critical importance in the elucidation of the pathogenesis of this destructive disease. It destroys alveolar walls by the breakdown of elastin causing irreversible enlargement of airspaces distal to the terminal bronchioles, usually without evidence of fibrosis.

ANIMAL MODELS: Mice are generally the choice of experimental models for research for the following reasons: both human and mice genomes have been sequenced, revealing that numerous genes appear to be unique to one species or the other, more than 10,000 genetic markers are mapped in the mouse providing useful landmarks for genetic studies; the ability to alter the genetic constitution of the mouse, either by inserting new genes or increasing the gene expression levels by transgenesis or by the removal or alteration of genes through gene replacement techniques e.g. knockout mice. Many hundreds of inbred strains and specialised stocks e.g. mutants, are available and thorough knowledge of anatomy, biology and physiology of mice, especially with regards to the immunological system and fast breeding at a relatively low cost, make these animals experimentally ideal.

The extensive knowledge of mouse biology and the huge genetic resources, including the ability to genetically manipulate mice, offers researchers the capacity to explore biological systems under physiological and pathological conditions (Hunninghake, Gadek et al. 1981, Brusselle, Bracke et al. 2006). Animal models, especially rodents are the preferred tools of experimental studies as studies in humans are either technically impossible or morally inconceivable. The major goals

of using murine models of COPD enable researchers firstly, to understand the cellular and molecular mechanisms involved in the disease and thus to strive for prevention or therapeutic treatment and secondly, to develop new specific drugs for COPD in order to reduce the acceleration progress of lung destruction and to improve both clinical and functional status of COPD patients, thus ensuring morbidity and mortality (Brusselle, Bracke et al. 2006).

For some time now in mice, the tracheal instillation of tissue-degrading enzymes has been used to study the development of emphysematous lung lesions as well as the inhalation of tobacco smoke and other noxious stimuli to induce lung tissue damage, although the development of emphysematous-like lesions appear to be strain-dependent and several mouse strains with naturally occurring genetic mutations develop emphysema spontaneously without external stimuli, although a number of theses mutations lead to multi-system defects and are thus not restricted to pathology of lungs (Snider, Lucey et al. 1986, Shapiro 2000, Guerassimov, Hoshino et al. 2004, Bartalesi, Cavarra et al. 2005, Brusselle, Bracke et al. 2006). Gene targeted mice may also show signs of airspace enlargement, but is crucial to distinguish airspace enlargement due to the development of abnormal lung morphogenesis from adult emphysema, which is characterised by the destruction of mature alveoli (Mahadeva and Shapiro 2002, Brusselle, Bracke et al. 2006). Here we describe in more detail a variety of murine models of COPD with reference to emphysema.

Pulmonary emphysema: Protease/antiprotease imbalance model: An imbalance between proteases and their inhibitors is believed to play an essential role in the development of pulmonary emphysema. This imbalance may occur either by

an excessive release of proteases by inflammatory cells and resident lung cells, or by a reduced synthesis or increased breakdown of antiproteases (Brusselle, Bracke et al. 2006). The protease/antiprotease hypothesis of emphysema was first proposed 40 years ago, based on the observations that smokers with a deficiency of Alpha-1-antitrypsin (α1-antitrypsin) were at increased risk for pulmonary emphysema and that intratracheal administration of papain, a plant protease, leads to emphysema in experimental animals (Gross, Pfitzer et al. 1965, Brusselle, Bracke et al. 2006).

Since the initial experiments of Gross; a variety of proteases have been instilled into the lungs of animals. In mice, the most consistent and impressive airspace enlargement has been by the intratracheal instillation of porcine pancreatic elastase (Valentine, Rucker et al. 1983, Brusselle, Bracke et al. 2006). The development of emphysema after instillation of human neutrophil elastase has also been described in mice (Lafuma, Frisdal et al. 1991, Brusselle, Bracke et al. 2006). These rather crude and acute instillation models can be useful in determining the capacity of a protease to cause emphysema, and to study downstream events such as alveolar repair. However, these instillation models have some drawbacks; they cannot be used to explore any upstream events; they cannot give any information about which proteases are involved in the pathogenesis of emphysema and lastly, it is difficult to extrapolate the findings on the acute effects of elastase instillation to the slowly progressive chronic onset of the disease in humans.

Transgenic mice model: Transgenic mice have artificially introduced alterations in their genome, resulting in expression or overexpression of the gene product of interest. These transgenic ‘gain-of-function’ models have added further

proof to the protease/antiprotease imbalance hypothesis (Brusselle, Bracke et al. 2006).

Mice that overexpressed human interstitial collagenase in their lungs spontaneously developed pulmonary emphysema (D'Armiento, Dalal et al. 1992, Brusselle, Bracke et al. 2006). Recently it has been shown that matrix metalloproteinase -1 (MMP-1) generated this emphysematous phenotype via the degradation of type III collagen (Shiomi, Okada et al. 2003, Brusselle, Bracke et al. 2006). A major disadvantage of this kind of model is that the gene of interest is also expressed throughout organ development and growth, which makes it impossible to separate developmental abnormalities from the structural injury in adult lungs that defines emphysema. This problem of constitutive expression of transgenes can be overcome by the construction of inducible transgenic expression models. Induced overexpression of interleukin 13 (IL13) or interferon-γ (IFN-γ) into the lung of mice causes a phenotype that mirrors human COPD (Wang, Zheng et al. 2000, Zheng, Zhu et al. 2000). In both models, the overexpression of these inflammatory cytokines was associated with an increased expression of MMPs and cysteine proteases (cathepsins). The emphysematous changes were partly inhibited after treatment with MMP-inhibitors or cysteine protease inhibitors. These data further establish the role of proteases in lung tissue destruction, a hallmark of pulmonary emphysema (Brusselle, Bracke et al. 2006).

Mahadeva and Shapiro reviewed data on several naturally occurring mutant mice that spontaneously developed emphysema due to genetic abnormalities. In some of these mouse strains, airspace enlargement is thought to be the result of a disturbance

in the balance between proteases and their inhibitors. For example, the pallid mice have a deficiency in α-1 antitrypsin and thus reduced elastase inhibitory capacity and spontaneously develop emphysema late in life (Martorana, Brand et al. 1993, Mahadeva and Shapiro 2002, Brusselle, Bracke et al. 2006). These histological changes are parallel with a decrease in lung elastin, but there is no alternation in the bronchial alveolar lavage cell (BAL) population (de Santi, Martorana et al. 1995). Chronic exposure to cigarette smoke significantly accelerated parenchymal destruction in pallid mice (Cavarra, Bartalesi et al. 2001, Takubo, Guerassimov et al. 2002, Brusselle, Bracke et al. 2006). This is probably related to the diminished antiprotease capacity in these mice, and correlates with the enhanced risk for pulmonary emphysema in humans with α-1 antitrypsin deficiency. Low levels of α-1 antitrypsin have also been demonstrated in tight skin mice. These mice develop emphysematous lesions at 2-4 weeks of age (Gardi, Cavarra et al. 1994, Keil, Lungarella et al. 1996, O'Donnell, O'Connor et al. 1999).

Oxidant/antioxidant imbalance model: Cigarette smoke contains high concentrations of reactive oxygen species (ROS). Increased levels of ROS in the airways due to cigarette smoking, originate directly from oxidants in cigarette smoke, but also indirectly from the release of ROS by infiltrating macrophages and neutrophils (Pryor and Stone 1993, Rahman and MacNee 1996, MacNee 2001). This excess of ROS disturbs the balance between oxidants and antioxidants result in oxidative stress (Bowler and Crapo 2002, Brusselle, Bracke et al. 2006). Oxidative stress may be important in different aspects of the pathology of COPD, since it could amplify the inflammatory responses, induce apoptosis, impair the function of

protective antiproteases and reduce the activity of corticosteroids in the treatment of COPD (Barnes, Shapiro et al. 2003, Brusselle, Bracke et al. 2006).

In other mouse models, it has been demonstrated that cigarette smoke induces oxidative stress (Brusselle, Bracke et al. 2006). Mice exposed to acute cigarette smoke showed a transient, but significant decrease in antioxidant capacity (TEAC: Trolox Equivalent Antioxidant Capacity) with a decrease in protein thiols and in ascorbic acid in BAL (Cavarra, Bartalesi et al. 2001). Cigarette smoke strongly affects the glutathione metabolism, which is an important feature of the antioxidant defence in the lung (Cantin, North et al. 1987, Teramoto, Uejima et al. 1996, Brusselle, Bracke et al. 2006). The crucial protective role of antioxidant system has been demonstrated in mice with a targeted disruption of the Nuclear factor, erthythroid-derived 2, like 2 (Nrf2), a redox-sensitive transcription factor that is involved in the regulation of many detoxification and antioxidant genes (Rangasamy, Cho et al. 2004, Brusselle, Bracke et al. 2006). Disruption of the Nrf2 gene in mice led to earlier-onset and more extensive cigarette smoke-induced emphysema compared with wild type animals. Moreover, emphysema in Nrf2-deficient mice exposed to cigarette smoke for 6 months was associated not only with an increased level of markers of oxidative stress, but also with more pronounced BAL inflammation and with an increased number of apoptotic alveolar septal cells (Rangasamy, Cho et al. 2004). This experimental emphysema model in Nrf2-deficient mice provides a clear link between excessive oxidative stress due to an impaired antioxidant system, and an increased inflammation, apoptosis and worsened emphysema.

Pulmonary repair process/airway remodelling: Long-term exposure to toxic gases and particles, mostly cigarette smoke, is the primary cause of COPD. Host defences against these stimuli include innate immune responses (mucociliary clearance, epithelial repair and the acute inflammatory response) and adaptive immune responses (humoral and cellular components). Both types of response are associated with a repair process that remodels damaged tissue by restoring the epithelium and microvasculature and by adding connective-tissue matrix in an attempt to return the tissue to its previous state (Hogg 2004, Hogg, Chu et al. 2004, Brusselle, Bracke et al. 2006).Unfortunately, this repair process often contributes to the action of chronic inflammation with the complexity to pathological changes leading to COPD (Siafakas and Tzortzaki 2002, Brusselle, Bracke et al. 2006). Cigarette smoke is known to inhibit human bronchial epithelial cell repair processes, though normally the epithelium has a tremendous capacity to repair itself following injury (Knight and Holgate 2003, Brusselle, Bracke et al. 2006).

Several groups have shown that the transforming growth factor-β (TGF-β), an anti-inflammatory cytokine, is involved in airway repair (Romberger, Beckmann et al. 1992, Hodge 2001, Brusselle, Bracke et al. 2006). In an elastase-induced murine model, lesions stabilise after the acute phase of tissue damage and repair (Brusselle, Bracke et al. 2006). Following endotracheal administration of elastase, expression of elastin in the lungs increased, resulting in lung elastin levels which were 30% higher than controls 8 weeks after challenge (Valentine, Rucker et al. 1983, Brusselle, Bracke et al. 2006). McGowan reported that retinoic acid receptor-γ (RARγ) knockout mice develop characteristics of emphysema, suggesting a role for retinoic acid (RA) in the generation and repair of pulmonary alveoli (McGowan 2002,

Brusselle, Bracke et al. 2006). Although Fujita et al reported that exogenous applied retinoic acid fails to reverse emphysema in adult mouse models; Ishizawa et al showed the opposite. In addition, they demonstrated that besides all-*trans*-retinoic acid, granulocyte colony-stimulating factor (GCSF) is able to promote lung tissue regeneration in this mouse model of pulmonary emphysema (Fujita, Ye et al. 2004, Ishizawa, Kubo et al. 2004, Brusselle, Bracke et al. 2006). Mao et al concluded that all-*trans*-retinoic acid could modulate the protease/antiprotease balance in a manner that may impact on emphysema pathogenesis. These results raise the possibility that the repair mechanism following injury may be manipulated by exogenous agents and might be important in the search for therapeutic agents of COPD (Mao, Tashkin et al. 2003, Brusselle, Bracke et al. 2006). In humans COPD is characterised by increased mucus production in large airways (mucous gland enlargement) as well as in bronchioles (goblet cell metaplasia) (Hogg 2004). In mice, submucosal glands were found only in the proximal regions of the trachea at the same density as in humans, but unlike in humans, these glands did not extend below the trachea. Epithelial mucous cells (i.e. goblet cells) in mice are mainly found in the proximal airways, but not in smaller airways such as terminal bronchioles (Evans, Williams et al. 2004, Brusselle, Bracke et al. 2006). Recently, goblet cell metaplasia/hyperplasia has been described in a murine model of COPD based on chronic cigarette smoke exposure. Comparing two strains of mice sensitive to oxidants, demonstrated that 75% of the animals in the C57BI/6J group showed a positive periodic acid Schiff (PAS) reaction of their large or middle size bronchi at 3 to 6 months smoke exposure; in contrast, in the DBA/2 group there were no PAS-positive animals at these time points (Bartalesi, Cavarra et al. 2005, Brusselle, Bracke et al. 2006). In C57BI/6J mice, the goblet cell metaplasia correlated with a positive reaction in the

airway epithelium on Immunohistochemical staining for IL-4, IL-13 and MUC5AC. However, goblet cell metaplasia is a non-specific phenomenon and can be induced by several other stimuli including ovalbumin challenge, LPS exposure and intratracheal instillation of neutrophil elastase (Yanagihara, Seki et al. 2001, Vernooy, Dentener et al. 2002, Shahzeidi, Aujla et al. 2003, Voynow, Fischer et al. 2004, Brusselle, Bracke et al. 2006). These in vivo models are valuable tools to further unravel the mechanisms involved in goblet cell metaplasia in COPD (Brusselle, Bracke et al. 2006).

Apoptosis model: Retamales et al showed that smokers who developed severe emphysema had an increase in the number of macrophages, T cell lymphocytes, neutrophils and eosinophils in their lungs compared with people who smoked similar amounts of cigarettes, yet maintained normal lung function (Retamales, Elliott et al. 2001, Brusselle, Bracke et al. 2006). This suggests that people who develop emphysema have an amplified inflammatory response to cigarette smoke, as explicitly mentioned in the definition of COPD by GOLD (Brusselle, Bracke et al. 2006). Also in murine models of chronic cigarette smoke exposure, there is a clear correlation between the magnitude of the pulmonary inflammation, comprising of macrophages, neutrophils and T cells and the development of emphysema (d'Hulst, Butterworth et al. 2004, Guerassimov, Hoshino et al. 2004, Brusselle, Bracke et al. 2006). Thus in both humans and mice, the traditional hypothesis for the pathogenesis of emphysema is that cigarette smoke induces an (exaggerated) influx into the lungs of the inflammatory cells that release ROS and proteases, causing the degradation of matrix with subsequent loss of attachment and death of structural cells (Brusselle, Bracke et al. 2006). However, at least three recent studies in mice demonstrated the

development of emphysema, despite a remarkable lack of inflammation (Kasahara, Tuder et al. 2000, Aoshiba, Yokohori et al. 2003, Petrache, Natarajan et al. 2005, Brusselle, Bracke et al. 2006).

The first model used a single intratracheal injection of active caspase-3 was used to induce emphysematous changes (Aoshiba, Yokohori et al. 2003, Brusselle, Bracke et al. 2006). This study provides direct evidence that in the alveolar wall apoptosis causes pulmonary emphysema, even without the accumulation of inflammatory cells (Tuder, Petrache et al. 2003). In the second model, the intravascular administration of a vascular endothelial cell growth factor receptor -2 (VEGF-2) blocker also generated non-inflammatory emphysema (Kasahara, Tuder et al. 2000). Vascular endothelial cell growth factor (VEGF) is indeed a growth factor required for endothelial cell survival and blocking VEGF leads to apoptosis of endothelial cells (Alon, Hemo et al. 1995). Chronic VEGF-2 blockage caused alveolar septal cell apoptosis and airspace enlargement (Brusselle, Bracke et al. 2006). Petrache et al. reported that ceramide a second messenger lipid, appears to be crucial mediator of alveolar destruction in emphysema. Indeed, intratracheal instillation of ceramide in naïve mice induced emphysema, while inhibition of enzymes controlling de novo ceramide synthesis prevented alveolar cell apoptosis and emphysema caused by blockade of the VEGF receptors in both mice and rats (Petrache, Natarajan et al. 2005, Brusselle, Bracke et al. 2006). These experimental observations in mice are in agreement with studies showing that smokers with emphysema have a high levels of apoptosis compared to smokers without emphysema (Kasahara, Tuder et al. 2000, Majo, Ghezzo et al. 2001, Yokohori, Ashiba et al. 2004). Also in smoke-exposed mouse lungs, an increase in apoptotic cells (epithelial cells and macrophages)

demonstrated that this was associated with enhanced expression of FasL and caspases, suggesting that cigarette smoke-induced apoptosis is mediated by a Fas/FasL death receptor apoptotic pathway (Rangasamy, Cho et al. 2004). Both models put forward a new concept in the pathogenesis of emphysema, whereby apoptosis and initial loss of epithelial or endothelial cells can trigger matrix destruction and alveolar space enlargement (Brusselle, Bracke et al. 2006). This contrasts with the traditional hypothesis of cigarette-smoke induced pulmonary inflammation leading to protease-mediated destruction of alveolar walls. Of course, both hypotheses on the pathogenesis of emphysema are not mutually exclusive and it is becoming progressively apparent that both excessive proteolysis (due to the protease/antiprotease imbalance) and apoptosis of epithelial and endothelial cells in the lungs interact and enforce each other's destructive potential (Brusselle, Bracke et al. 2006).

Elastase-induced model: Porcine pancreatic elastase (PPE) instillation was introduced over 40 years ago by Gross et al to develop a model of emphysema in hamsters (Gross, Pfitzer et al. 1965, Fehrenbach 2006). The establishment of this animal model had a great impact on the development of proteinase-antiproteinase concept of emphysema formation (Shapiro 1995, Fehrenbach 2006). Since then, this model has been adapted by many others. The appeal of this model is that it has produced the most consistent airspace enlargement in rodents, guinea pigs, dogs and primates (Fig 1.5) (Kuhn, Yu et al. 1976, Snider, Lucey et al. 1986, Shapiro 2000).

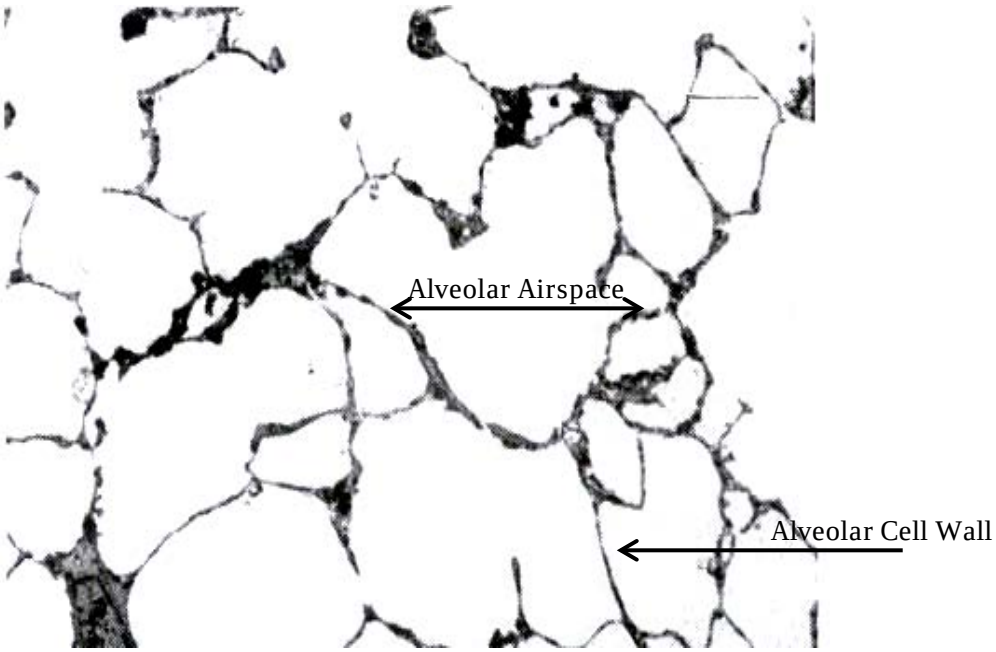

Fig 1.5: COPD/Emphysema Elastase-induced Rat model. Alveolar airspace and cell walls are large and thin in appearance (Massaro and Massaro 1986).

The instillation of PPE results in rapid and significant airspace enlargement followed by acute neutrophil and subacute macrophage accumulation within the lung (Heemskerk-Gerritsen, Dijkman et al. 1996, Shapiro 2000, Fehrenbach 2006). Airspace enlargement continues over the first month after instillation and then stabilizes. Elastin content initially decreases, but elastin messenger RNA (mRNA) expression and elastic fibre disorganisation is observed within weeks (Shapiro 2000). However, the desired effect of elastase is frequently limited to a narrow window of dosage, below which no significant loss of alveoli is observed; whereas a higher dose may result in severe pulmonary haemorrhage and high mortality (Hayes, Korthy et al. 1975, Busch, Lauhala et al. 1984, Fehrenbach 2006). Work carried out by Massaro and Massaro with an elastase-induced rat model observed an increase in the mean alveolar volume by approximately 240%. This was associated with a

decrease in the total number of alveoli by approximately 45% after elastase treatment of rat lungs. Using a selector approach for quantification, total alveolar surface area decreased insignificantly by only 5%. Massaro and Massaro explained this discrepancy through the low elastic recoil of elastase-treated lungs (inferred from the increased fixed lung volume per body weight) which allowed an overexpansion of the lung (Massaro and Massaro 1997, Fehrenbach 2006).

Elastase-induced emphysema remains a useful model of emphysema since it is relatively simple to perform and replicate many aspects of the disease. Of course, exposure to cigarette smoke may cause a variety of other abnormalities not observed in this model and indeed other models of emphysema.

Glucocortocoids (Dexamethasone-induced) mouse model: The process of alveolar formation can be influenced by hormones such as glucocorticoids and RA. It has been demonstrated in postnatal rats and foetal murine lungs that dexamethasone can inhibit secondary septation (the microvascular maturation with fusion of double capillary layers into single medial layers of alveolar lumens of the septum and thinning of alveolar cell walls by apoptosis) in these lungs with differing doses of RA have varying effects on proliferation and differentiation (Fig 1.6).

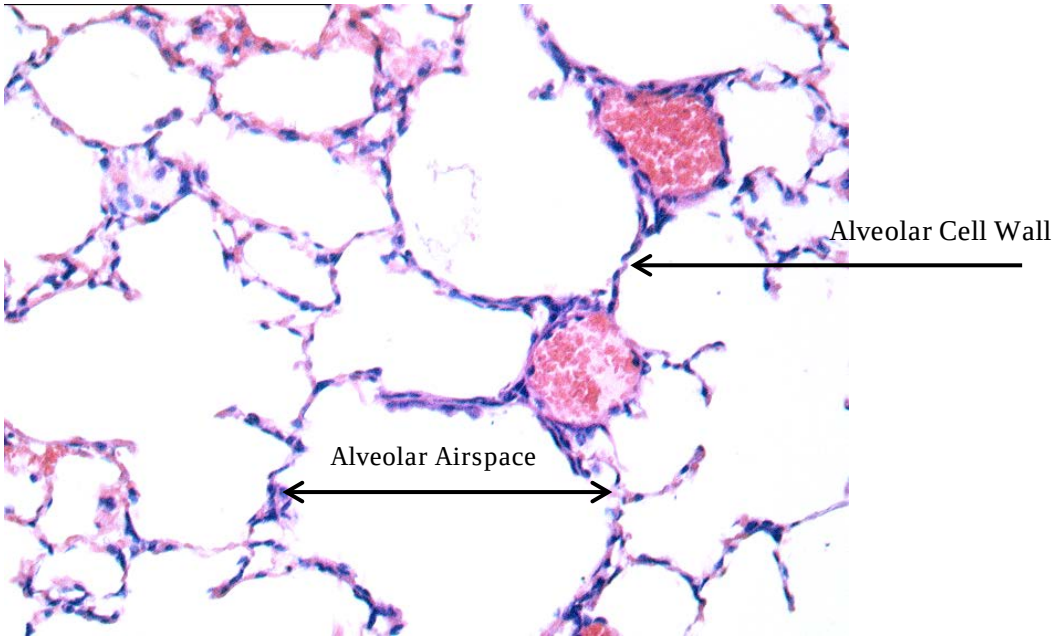

Fig 1.6: COPD/emphysema dexamethasone-induced mouse model. As previously mentioned in the rat elastase-induced model, alveolar airspace and cell wall are large and thin in appearance.

RA has been shown to induce septation long after the administration of dexamethasone, used to induce injury during the second stage of septation (Massaro and Massaro 1986, Cilley, Wesley et al. 1987, Tschanz, Damke et al. 1995, Massaro and Massaro 1996, Chinoy 2003). High dosage levels of dexamethasone lower the level of retinoic acid receptor-beta (RARβ) whereas oxygen treatment, which also has the capacity to inhibit septation, has the opposite effect by increasing the levels of RARβ (Grummer and Zachman 1995).

Normally, glucocorticoids are used as critical regulators for the surfactant system in late foetal life and antenatal glucocorticoids are given to women at risk of preterm delivery, thereby decreasing the chances of respiratory distress system (RDS) in the

new born. Glucocorticoids are potent antiviral and anti-inflammatory agents. They are known to inhibit cell division as seen in lungs and are known to inhibit apoptosis (Morishige and Joun 1982, Jobe and Ikegami 1998). Furthermore, serum concentration of active glucocorticoids rises as the second phase of septation accelerates into the stage of alveolar thinning of the wall. This puts forward the idea that a rise in concentration of glucocorticoids may initiate the end of alveolar septation and cause acceleration of alveolar thinning of the cell wall and the remodelling of alveolar wall from a double capillary to a single capillary system (Burri 1985).

Experimentally, exogenous dexamethasone (dex) prevents normal formation of alveoli in rats and mice (Massaro, Teich et al. 1985, Tschanz, Damke et al. 1995, Maden and Hind 2003, Hind and Maden 2004). In addition to blocking septation, dex dosing dramatically accelerates alveolar thinning of the walls and changes the composition of the wall (Massaro and Massaro 1986). Within 2 days of dex-treatment, dex dosed pups have 20% thinner walls (reducing gas-exchange), 32% lower volume of lipid-laden fibroblasts and a 1.5 fold higher volume of ATII cells (Hind and Maden 2004). These experiments suggest dexamethasone treatment diminishes the replication of fibroblasts in alveolar walls and impairs the conversion of ATII cells to ATI cells, but the mechanism by which dex inhibits septation needs further exploration.

Limitations of using Murine COPD models: In vivo murine models can offer valuable information on several aspects of the pathogenesis and treatment of COPD, in particular, emphysema. However, as for other animal species, murine models of

COPD have several limitations. Firstly, no model can mimic the entire COPD phenotype since many models can only specifically mimic one trait of this disease e.g. enlargement of the pulmonary alveoli due to injury to the lung parenchyma (pulmonary emphysema). However, the pathogenesis of progressive and fixed airflow limitation due to chronic airway obstruction which defines COPD has not yet been thoroughly addressed in murine models (Hogg 2004, Brusselle, Bracke et al. 2006). Moreover, each murine models of emphysema has its own specific disadvantages. The most important disadvantages of the protease-induced emphysema, is the lack of significant inflammation and the absence of airway changes such as mucous cell metaplasia (Brusselle, Bracke et al. 2006). Although there is a significant inflammatory reaction in the cigarette smoke-induced emphysema, this murine model has also several specific limitations since there is a lack of standardised exposure and standardised morphometric analysis (March, Green et al. 2000, Brusselle, Bracke et al. 2006). Various smoking machines and various exposure regimens are used, but the choice of smoking regimen is often made arbitrarily (Rennard 2004, Brusselle, Bracke et al. 2006). In addition, breathing smoke generated by a machine is not the same as active smoking, but rather resembles passive smoking. Secondly, there are considerable differences in the respiratory physiology and anatomy between rodents and humans. In contrast to humans, rodents are obligate nose breathers and the submucosal glands in rodents are restricted to the trachea. During the embryonic stage of lung development, there are important species different from rodent and human lung morphogenesis including pulmonary lobation and bronchial branching (Warburton, Schwarz et al. 2000, Brusselle, Bracke et al. 2006). Critically, pulmonary emphysema in humans is classified according to the distribution of airspace enlargement within the acinus: in

the panacinar emphysema, as seen in patients with α_1-antitrypsin deficiency; enlargement and destruction of airspaces of the acinar unit are uniform, whereas in centriacinar emphysema, as seen in cigarette smokers; the airspace enlargement occurs primarily in three generations of respiratory bronchioles (Hogg 2004, Brusselle, Bracke et al. 2006). Rodents however do not have clearly defined respiratory bronchioles or distinct lobular architecture (March, Green et al. 2000, Brusselle, Bracke et al. 2006). These distinctions in anatomy have to be taken into account when extrapolating experimental data from murine models to human disease (Brusselle, Bracke et al. 2006).

1.5: THE IMPORTANCE OF VITAMIN A IN LUNG DEVELOPMENT:

Vitamin A is essential for growth and development of cells and tissues. In its active form, RA controls the regulation for differentiation as a ligand of the retinoic acid receptors, RAR/RXR and is involved in the integration of cell formation. Vitamin A plays a substantial role, especially in the respiratory epithelium of lungs. During vitamin A deficiency, the incidence for diseases of the respiratory tract is considerably increased and repeated respiratory infections can be influenced therapeutically by vitamin A supplementation. In addition to the importance of this vitamin for lung function, vitamin A is also responsible for the development of many tissues and cells as well as for embryonic lung development. Recent studies proved that the control occurs by different expressions of retinoid receptors as well as by time-dependent changes of the vitamin A metabolism respectively via cellular vitamin A binding proteins (CRBP: cytoplasmic retinol binding protein; CRABP: cytoplasmic retinoic acid binding protein) (Fig 1.7).

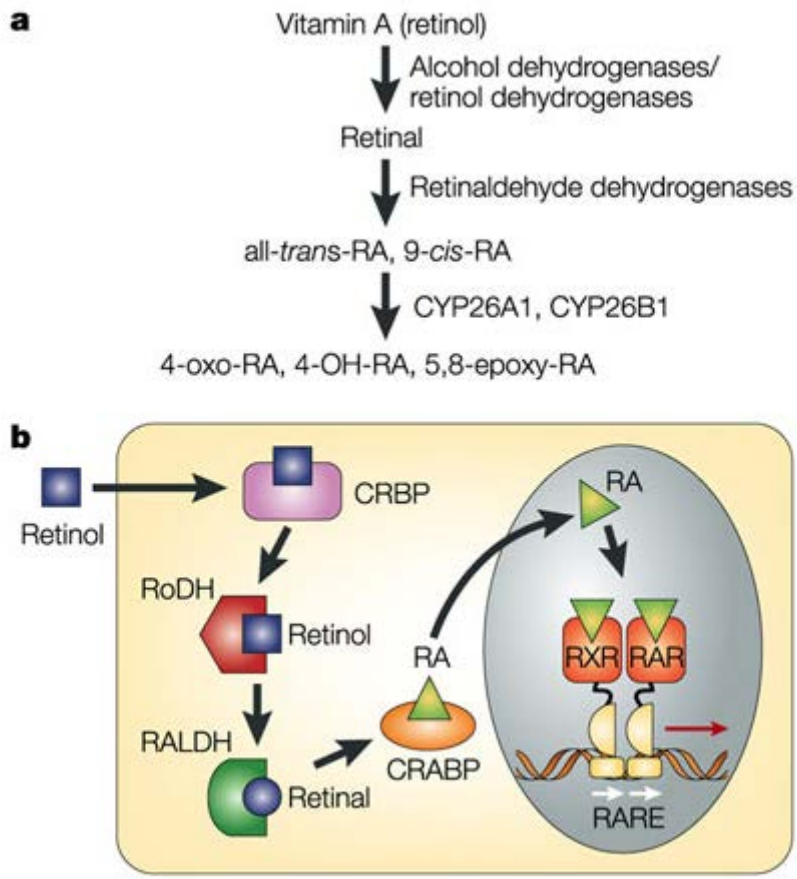

Fig 1.7: The metabolic pathway of vitamin A (retinol) conversion into the various forms retinoic acid (RA) and its three classes of enzymes (a). The cellular mechanism of retinoid; (b). Retinol is taken up from the blood and bound to CRBP (cellular retinol-binding protein) in the cytoplasm. The retinol dehydrogenase (RoDH0 enzyme metabolise retinol to retinal, then is metabolised to RA by the retinaldehyde dehydrogenases (RALDHs). RA is bound in the cytoplasm by CRABP (cellular RA-binding protein). RA enters the nucleus and binds to the RA receptors (RARs) and the retinoid x receptors (RXRs), which themselves heterodimize and bind to a sequence of DNA known as the RARE (RA-response element). This activates transcription of the target gene. Nature Reviews/Neuroscience 2002. **3:** 843-853

Previous studies using vitamin A deficient animal models have shown that disruption of retinoic acid (the active form of vitamin A) signalling may lead to lung agenesis (Cardoso and Whitsett 2008). This raises the possibility that in the absence of retinoic acid (RA) signalling, lung progenitor cells may not be specified or fail to expand.

Retinoic acid and its receptor agonists as therapeutic agents (Regenerative therapies): Currently, COPD which is a progressive disease is only managed symptomatically. So far, the most successful treatment available for COPD is lung transplantation. However, finding suitable donor material is very limited and therefore only available to a limited number of patients. One possible way to treat such disease would be to induce diseased lung tissue to “self heal” or “regenerate”. Unfortunately, a significant barrier to such regenerative therapies is that damaged adult lung alveolar tissue seems incapable of spontaneous repair. Therefore, understanding how to activate repair mechanisms would greatly improve the prospect of effective treatment and prognosis for patients.

RA has been demonstrated to promote alveolarization in both rat and mouse models, but other reports in both species show failure of RA to promote alveolarization (Massaro and Massaro 1997, March, Green et al. 2000). Oral therapy with All-*trans*-retinoic acid (tRA) in COPD has been reported and there has been evidence suggesting some respiratory improvement, therefore it has been considered as a candidate as a therapeutic agent for patients with COPD.

Another potential therapeutic agent for COPD is the granulocyte macrophage-colony stimulating factor (GM-CSF). GMCSF is a major regulator of both macrophage and neutrophil activation and survival in the lung; these cells are intimately linked to COPD (Fig 1.8). Experimental data using animal models indicate that neutralisation of GMCSF ameliorates experimental COPD and predicts therapeutic utility in treating stable COPD and treating exacerbations. As such, GMCSF represents an attractive therapeutic target for the treatment of COPD.

CHAPTER 2

MATERIALS AND METHODS

2.1: MATERIALS

ANIMAL HUSBANDRY AND TISSUE PREPARATION

All experiments were conducted in accordance with local ethics committee guidelines (Kings College London) under the requisition personal (piL 70/15855) and project licence (PPL70/4452) obtained from the Home Office.

2.1.1: WISTAR RAT BRED FOR VITAMIN A DEFICIENCY STUDIES. In order to test the importance and role of vitamin A for the development, structure and function of the respiratory system, a vitamin A deficient rat postnatal model (Wistar Bred from 3 months) was generated by replacing a normal laboratory chow diet with a commercially available diet that was lacking vitamin A (retinoids) with a SDS nutritional one. All animals were given access to water and a 12-hour cycle of light and darkness. The animals were sustained on this diet for a period of 6 months to 1 year. Within this time frame, body weight and general health were monitored. After sacrifice, samples of blood serum and liver were taken for high performance liquid chromatography (HPLC) to confirm a reduction of retinoids in the animal's system.

2.1.2: SWISS TO MOUSE BRED FOR REGENERATIVE STUDIES. Outbred Swiss (TO) mice (Harlan, UK) were used to generate structural emphysematous by the use of the glucocorticoid, dexamethasone. All animals were given access to water and laboratory chow ad libitum and a 12-hour cycle of light and darkness. For consistency, the day of birth was deemed as postnatal day 1 (P1). In dexamethasone experiments, litters were reduced at birth to no more than 21 pups in an attempt to

reduce variation in developmental stages. For retinoic acid and its receptor agonists dosing experiments, postnatal age-matched female mice were used.

2.1.3: TISSUE PREPARATION. Animals were culled by neck dislocation, the diaphragm was punctured and the thorax opened by removal of the ventral thoracic wall. The heart and the trachea were cut at the level of larynx. The trachea and the lungs were carefully removed, ensuring that no lacerations to the surface of the lungs. For histology, lungs were carefully dissected to maintain morphology. The trachea was cannulated and tied firmly in place, infused with fresh 4% formalin at a standard inflation pressure of 20cm H_20 for at least 10 minutes. The cannula was then removed and the trachea ligated. The lungs were stored in 4% formalin until required. Processing of samples were as follows (Table 2.1.1).

TABLE 2.1.1: PROCESSING PROCEDURES FOR PARAFFIN WAX EMBEDDED TISSUE.

SOLUTIONS	PERCENTAGE (%)	TIME (hrs)
Industrial methylated spirit: (IMS)	30%	2hrs
Industrial methylated spirit: (IMS)	70%	2hrs
Industrial methylated spirit: (IMS)	90%	2hrs
Industrial methylated spirit: (IMS)	100% (Absolute)	2hrs
Industrial methylated spirit: (IMS)	100% (Absolute)	2hrs
Industrial methylated spirit: (IMS)	100% (Absolute)	2hrs
IMS/Xylene	50-50	2hrs
Xylene	100% (Absolute)	2hrs
Xylene	100% (Absolute)	2hrs
Xylene	100% (Absolute)	2hrs
Melted wax: (65-72°C)	100%	2 -4hrs

2.1.4: GENERAL SOLUTIONS. The following standard buffer blocks and reagents made for the techniques described below are referred to in the text by the name or abbreviation below. All reagents were made with UHQ water.

HISTOLOGY/IMMUNOHISTOCHEMISTRY/IMMUNOFLUORSCENCE SOULTIONS

10X PHOSPHATE BUFFERED SALINE (PBS: Sigma, Poole. UK)

80g Sodium Chloride: VWR International, UK

14.4g Sodium Phosphate: Sigma-Aldrich, Poole. UK

20g Potassium Chloride: VWR International, UK

2.4g Potassium Phosphate: Sigma-Aldrich, Poole. UK

Ingredients were mixed in 1 litre of UHQ water to pH7.4

ABSOLUTE XYLENE (2.5L): VWR International, UK

INDUSTRIAL METHYLATED SPIRIT 2.5L IMS) VWR International, UK

4% FORMALIN TISSUE FIXATIVE: 500ML

50ml Formaldehyde (W/V). VWR International, UK

50ml PBS (from 10% Stock solution: W/V)

Ingredients were mixed with 400ml of UHQ water

HAEMATOXYLIN AND EOSIN (H&E) FOR GENERAL MORPHOLOGY

500ml MAYER'S HAEMATOXYLIN: Ready-to-use. VWR International, UK

1% EOSIN (Eosin yellowish. Ready-to-use). Raymond A Lamb, UK

ALCIAN BLUE PERIODIC ACID SCHIFF'S REAGENTS (ABPAS) FOR ACIDIC AND NEUTRAL GLYCOPROTEINS

12.5g Alcian Blue in 500m UHQ water (2.5%) VWR International, UK

5g Periodic Acid in 500ml UHQ water (1%) Sigma-Aldrich. UK

Schiff's Reagent (Ready-to-use) VWR International, UK

5M TRIS BUFFERED SALINE STOCK SOLUTION (TBS): pH7.54

30.20g Tris VWR International, UK

42.86g NaCl VWR International, UK

Ingredients mixed with 500ml of UHQ

2% BOVINE SERUM ALBUMIN BLOCK (BSA): 200ml

4g BSA (12% W/V). Sigma-Aldrich. Poole, UK

20ml TBS 10X stock solution

2ml 10% Azide Sigma-Aldrich. Poole, UK

Ingredients were mixed with 180ml UHQ water

1% TBS/TWEEN WASH (TBS-T): 500ml

50ml 10X TBS

5ml Tween 20 Sigma-Aldrich, Poole. UK

Ingredients were mixed with 450ml of UHQ water

ANTIGEN RETRIEVAL BUFFER SOLUTION

CITRATE ACID pH6.0

8ml 1M Citrate Acid BDH/MERCK, UK

Mixed with 792ml UHQ water

SDS GEL AND WESTERN BLOT SOULTIONS

SDS-PAGE SOLUTIONS

500ml NuPAGE MES SDS RUNNING BUFFER (20X) Invitrogen Life Technologies. UK

25ml SDS-Mes Running Buffer

475 UHQ water

500ml NuPAGE TRANSFER BUFFER (20X) Invitrogen Life Technologies. UK

25ml Transfer Buffer

50ml-100ml Methanol VWR International

Ingredients were mixed with 425 or 375ml UHQ water

WESTERN BLOT MATERIALS AND SOLUTIONS

Filter paper VWR International, UK

Nitrocellulose paper GE Healthcare, UK

PREPARATION OF WESTERN BLOT

Soak sponges in transfer buffer until saturated, remove any air bubbles. Removing air bubbles is essential as they block the transfer of proteins if they are not removed. Cut transfer membrane and filter paper to the dimensions of the gel. Place nitrocellulose in a weigh boat containing premade transfer buffer solution. Assemble the western blot "sandwich" as follows in XCell blot module and is summarised in a simple diagram (Fig 2.1.1):

- 2-3 soaked sponges
- 1-2 wetted filter papers
- 4-12% gel containing varying MW protein samples (make sure no air bubbles are present)
- Soaked nitrocellulose membrane (again make sure no air bubbles are present)
- 1-2 wetted filter papers
- 2-3 soaked sponges

Fig 2.1.1: Western Blot is the transfer of proteins on a gel to nitrocellulose membrane in order to perform immunoblotting to detect the desired protein on the membrane. (*Abcam, Cambridge. UK*)

BLOCKING SOLUTION FOR NITROCELLULOSE MEMBRANE.

2% Powdered milk or BSA

Add to TBST buffer (milk) mix well and filter (prevents spotting where tiny dark grains will contaminate the blot during development).

2.2: METHODS: EXPERIMENTAL PROCEDURES.

2.2.1: DEXAMETHASONE AND RETINOIC ACID DOSING

Dexamethasone (Sigma, Poole. UK) was dissolved in PBS and given as a 10µl subcutaneous injections at a concentration of 40µg/ml. This was administered for 10 days (from P4 to P13) control mice received PBS. On day 46, various retinoid ligands were administered for 10 days with a 2 day break on P40 and P41. The retinoids were dissolved at a concentration of 2mg/ml in dimethylsulphoxide/peanut oil (1:1) and a dose of 2mg/ml was administered by intraperitoneal injection. A 10-fold higher dose of all-*trans*-retinol was administered because this precursor of tRA is known to be less effective. Control mice received DMSO/peanut oil only. tRA was obtained from Sigma; the retinoic acid receptor agonist ligands (RARs) were obtained from CIRD Galderman (Sophia Antipolis, France): and the retinoid X receptor agonist ligand (RXR) was obtained from Hoffman-La Roche's (Basel, Switzerland). Animals were kept for 4 weeks after the end of the retinol dosing until week 13 and sacrificed for regeneration analyses (Fig 2.2.1 and 2.2.2).

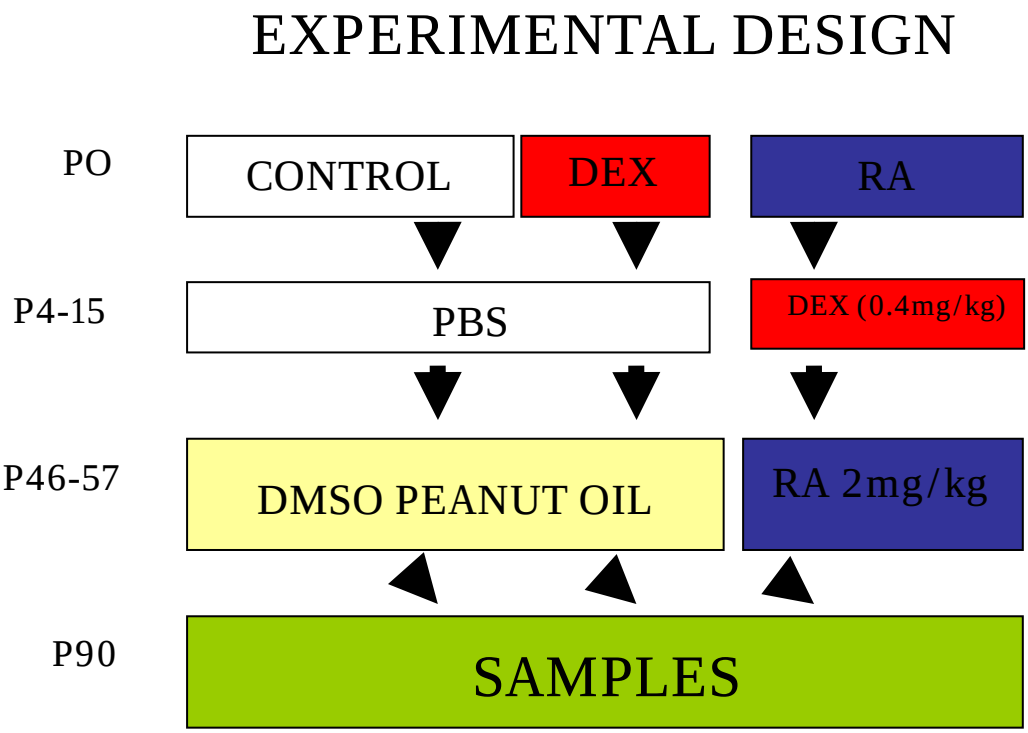

Fig 2.2.1: Experimental design for dexamethasone postnatal mouse model to induce emphysematous-like features and retinoic acid (RA) to promote regeneration.

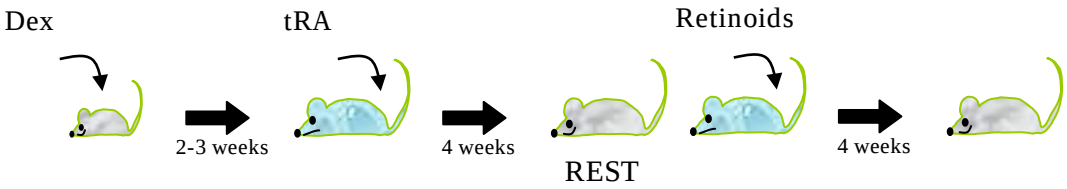

Fig 2.2.2: Experimental design for retinoid-selective ligand agonists employed to induce alveolar regeneration.

2.2.2: MORPHOLOGY AND MORPHOMETRICS

MORPHOLOGY: Animals were weighed and their lungs were carefully removed and intubated with a 22 gauge cannula. Lungs were inflated to a standard pressure of 20cm and H_2O with 4% formalin for 48hrs. Lungs were washed in water and placed in 70% IMS (alcohol); their volumes were measured by fluid displacement. Lungs were then processed in ascending alcohols, embedded in paraffin wax and sectioned at 5µm thick. These sections were stained with H&E for morphology analysis.

MORPHOMETRICS: Sections were analysed on light microscope linked to a digital imaging system. Images obtained were acquired at 10x objective and processed using ImageProPlus analysis software (Data Cell Ltd; Finchampstead. UK). Areas of alveolar tissue were selected and calculated (Fig 2.2.3). Five non-overlapping fields were measured from each section of animal group. Gas exchange surface area was calculated from values in L_m. All data was expressed as mean ± standard error (SEM). Groups were statistically compared by Analysis of variance (ANVOA) test followed by the Bonferroni correction test using GraphPad Prism5 software.

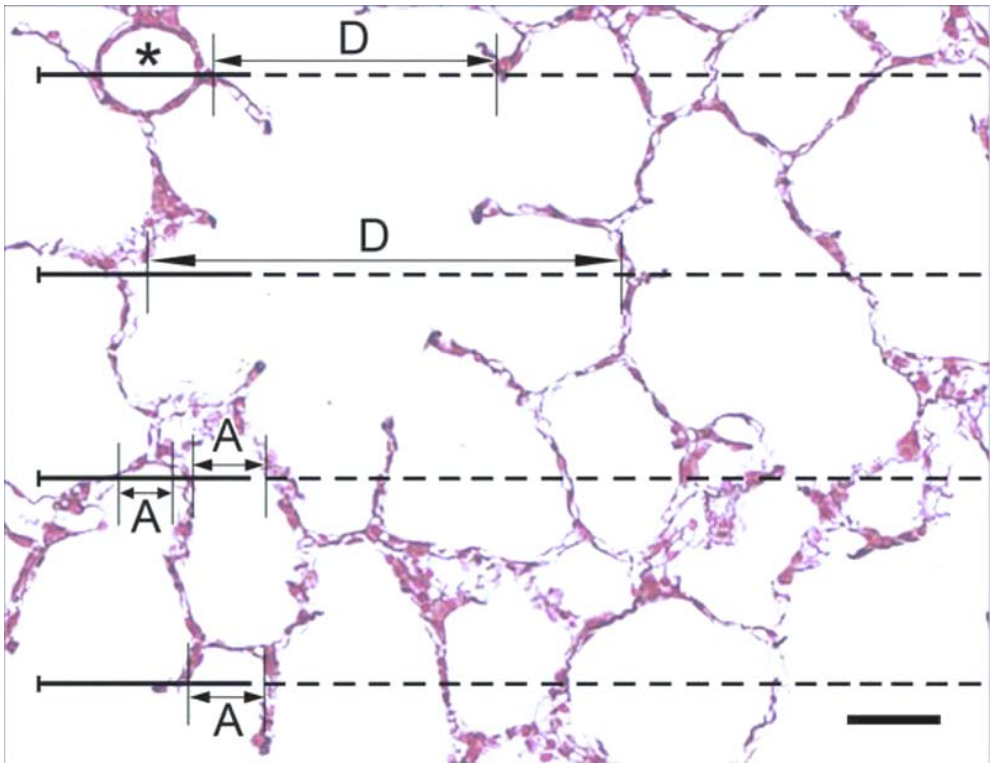

Fig 2.2.3: Representative diagram of the mean linear intercept (Chord) length (L_m) based on the intercept distribution. Test line segments are localised at the left, followed by a dashed line towards the right. Each time the test line intersects the alveolar wall, the distance (chord length) to the next wall is measured. Boundaries of measurements within alveolar airspace (A) or alveolar and ductal airspace together (D) are marked by arrows. * Blood vessel. Scale bar = 50µm (Knudsen, Weibel et al. 2010).

2.2.3: IMMUNOHISTOCHEMICAL AND IMMUNOFLUORESCENT STAINING FOR PARAFFIN WAX TISSUE SECTIONS

4% formalin-fixed 5µm paraffin sections were cut and mounted onto superfrost plus microscopic slides and incubated overnight in a 60° oven before proceeding to stain the next day. The sections were treated as follows: sections were dehydrated in two

pots of absolute xylene then dehydrated in four pots of industrial methylated spirit (IMS) for approximately 3 minutes. Sections were rehydrated in distilled water and treated with hydrogen peroxide (H_2O_2) to quench endogenous peroxidase, for 10 minutes. Preheated citric acid buffer solution (pH 6.0) was used as an antigen retriever to unmask formalin meshwork covering antigens of interest. Sections were allowed to cool down in cold tap water and incubated with 2% bovine serum albumin (BSA) to block endogenous biotin and incubated with a primary antibody of interest at room temperature, overnight. 5 minute washes with 1% tris buffer saline/tween (TBS-T) were applied before incubating sections with secondary biotinylated antibodies for 30 minutes (Dako UK). Sections were incubated with streptavidin biotin complex horseradish peroxidase (StreptABC-HRP. Vector Laboratories. UK), for a further 30 minutes. Immunoreactivity was visualised using a peroxidase substrate; 3.3-diaminobenzidine (DAB. Sigma Aldrich, Poole UK). After 10 minutes of DAB incubation, sections were counterstained with Mayer's haematoxylin (nuclear Counterstain) for 30 seconds. Antibodies and dilution factors for IHC/IF and immunoblots are listed in Table 2.2.1

Table 2.2.1: Antibodies and dilution factors for IHC and Immunoblots

Primary Antibody	IHC Dilution	WB Dilution	Secondary Antibody	Dilution IHC/WB
SP-C	1:100	1:1000	Rabbit	1:200 – 1:2000
CC10	1:2000	1:20,000	Goat	1:200 – 1:2000
PCNA	1:100	1:1000	Mouse	1:200 – 1:2000
TTF-1	1:50	1:5000	Mouse	1:200 – 1:2000
MUC2	1:100	1:1000	Mouse	1:200 – 1:2000
MUC5AC	1:2000	1:1000	Rabbit	1:200 – 1:2000
MUC5B	1:100	1:1000	Mouse	1:200 – 1:2000
MUC7	1:750	1:1000	Mouse	1:200 – 1:2000

Immunostained cells were counted in the alveolar epithelia or bronchiole epithelia in at least ten non-overlapping high power field (HPFs 400X magnification) per animal group.

2.2.4: SDS-PAGE ELECTROPHORESIS FOR MUCIN PROFILE

SDS –PAGE electrophoresis was performed on all samples obtained from the control and treated animals. Equal volumes (15µl) of heat-denatured whole lung protein samples were applied to each lane and electrophoresed on precast 4-12% Bis-Tris gels, according to the manufacturers protocol, in MES-SDS running buffer (Fig 2.2.4). Proteins in gels were stained with 0.2% Coomassie Brilliant Blue R20 in 25% methanol, 10% acetic acid and water for 30 minutes and then de-stained in 10%

acetic acid, until the gel background was clear. Glycoproteins in the gels were stained with periodic acid Schiff (PAS). Gels were fixed in 25% methanol and 10% glacial acetic acid for 1 h, after which the gel was washed in distilled water for 20 minute. The gel was then oxidized in 2% periodic acid for 15 minutes and then rinsed in distilled water. Schiff's reagent was added neat and incubated in the dark with gentle agitation for 30-60 minutes or until pink stained bands appeared. The Schiff's reagent was discarded and the gel was rinsed in distilled water to increase the stain intensity or in tap water to lighten the stain intensity.

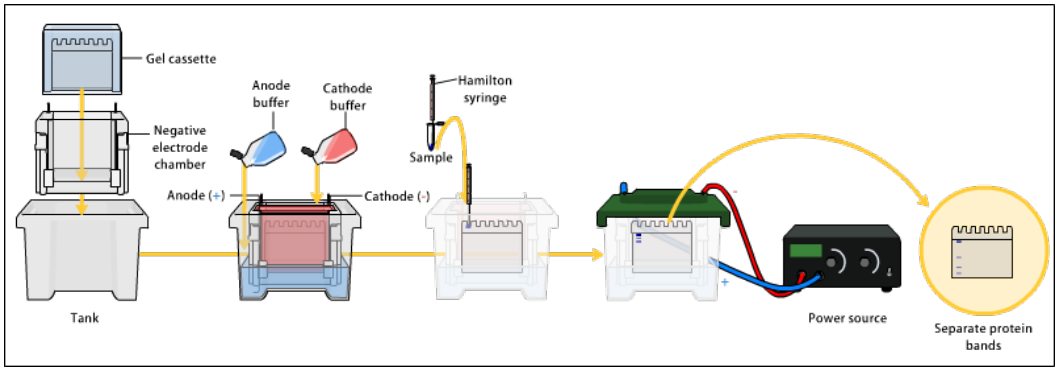

Fig 2.2.4: Sequence of SDS-PAGE electrophoresis. Samples were loaded onto 4-12% Bis-Tris gel and run in SDS-MES running buffer for approximately 32 minutes. *Abcam, Cambridge. UK*

SDS gels were photographed using a digital camera (Kodak DC290 Zoom Digital Camera; Anachem, Bedfordshire, UK) using the software Kodak Electrophoresis Documentation and Analysis System 290 (Anachem). Gels were scanned using the software, ImageJ (downloaded from National Institute of Health, http://rsb.info.nih.gov/ij). Lanes of PAS stained mucin glycoprotein bands were scanned and a lane profile plot was generated with peaks representing each mucin

band according to intensity. The area under each peak was measured and used to express the amount of mucin in PAS-stained mucin bands.

2.2.5: WESTERN BLOTTING. Immunoblotting onto nitrocellulose membranes was carried out according to a standard protocol. Membranes were blocked in a solution of TBST milk or with 2% BSA for 1 hr at room temperature according to the manufacturer's instructions. Membranes were then probed with a range of antibodies, diluted in 2% TBST milk or 2% BSA, for 1 hr at room temperature or overnight at 4°C, as recommended (Fig 2.2.5). Mouse monoclonal, Goat and Rabbit polyclonal antibodies were used to detect mucin glycoproteins MUC1, MUC2, MUC4, MUC5AC, MUC5B and MUC7 (Santa Cruz Biotechnology CA, USA. Development Hybrid Study Bank [DHSB], University of Iowa, USA. Labvision, Thermo Scientific. MI, USA. Sigma-Aldrich, Poole. UK and Ana-Spec! CA, USA). Rabbit and goat polyclonal antibodies, Surfactant protein-C (SP-C) and Clara cell 10 (CC10) (Santa Cruz Biotechnology CA, USA) were used to detect airway progenitor cells. A mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody was used to detect cell proliferation (DAKO Corporation, Denmark) and a mouse monoclonal thyroid transcription factor-1 (TTF-1) antibody (Labvision, Thermo Scientific. MI, USA) was used to detect Nkx2.1 regenerative cells in the alveolar and bronchiole epithelia (Fig 2.2.5 and 2.2.6).

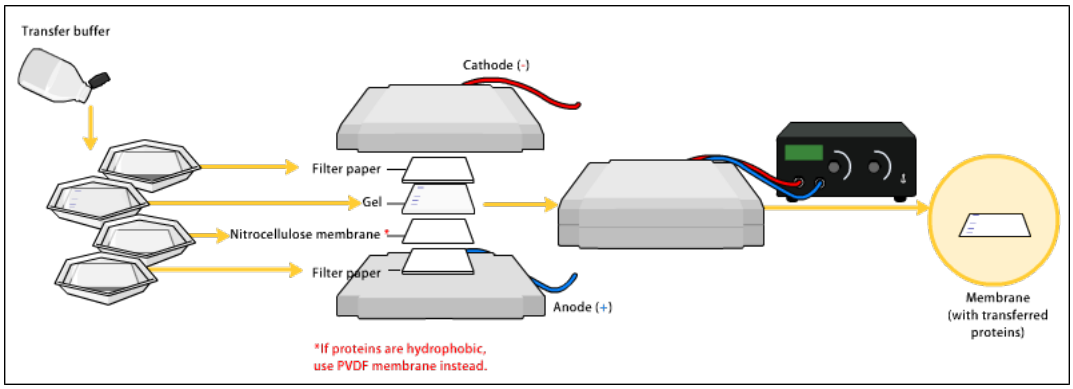

Fig 2.2.5: Sequence of principal of western blot. *Abcam, Cambridge. UK.*

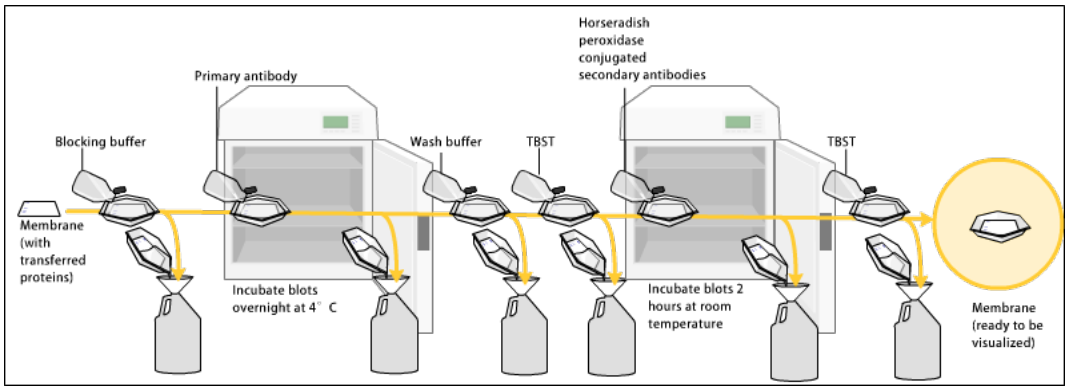

Fig 2.2.6: Sequence of detection of specific proteins via Immunoblot. *Abcam, Cambridge. UK.*

2.2.6: IMAGEJ AND IMAGELAB ANALYSIS. Intensity of protein bands on SDS gels were scanned using the software ImageJ (downloaded from National Institute of Health, http://rsb.info.nhs.gov/ij). Lanes of PAS stained mucin glycoprotein bands were scanned and a lane profile plot was generated with peaks representing each mucin band according to intensity. The area under each peak was measured and used to express the amount of mucin in PAS-stained mucin bands. Immunoblots were analysed using the software ImageLab for the gel doc; Chemi-

Doc (BioRad. Laboratories. UK). Images of bands of various samples were captured for quantitative analysis. Lanes of immunostained bands from each sample was measured according to intensity and density of the band to express the amount of protein in each group.

CHAPTER 3.1

MORPHOLOGY AND MORPHOMETRIC ANALYSIS OF POSTNATAL VAD RAT LUNGS

OBJECTIVE: In this study, we examined morphological and morphometric changes caused by a dietary deficiency of vitamin A in postnatal rats for up to one year. We first examined animals fed on this diet for 6 months and found the beginnings of morphology and morphometric changes. Using a histological staining technique designed to illustrate morphological changes and calculating surface area of alveoli; the mean chord intercept (L_m) parameter we were able to determine changes in 6 months VAD lungs compared with 6 months controlled lungs. Alveolar showed signs of airway expansion (surface area) with an increase in vascularisation in these animals. Further experimental analysis with animals fed for up to 1 year showed significant changes in morphology and morphometric analysis. Lungs of these animals showed emphysematous-like structures, a significant increase in surface area (Sa). Vascularisation had also increased and cell wall thickness of alveoli had dramatically decreased. This data provided evidence that a chronic reduction of dietary vitamin A severely affects structural development and the functional role of the lung.

3.1.1: INTRODUCTION: Postnatal lung development is characterised by stage of alveolarization and microvascular maturation (Frey, Egli et al. 2004). At birth, large gas exchanging saccules are still present and are linked by thick walls with a double capillary network. Postnatally, these saccules subdivide by secondary septa via the process of alveolarization. Numerous studies examining vitamin A have recognised its importance in influencing the development and growth of the respiratory system, especially during its critical postnatal period (Gross 1990, Frey et al. 2004).

VITAMIN A IN RESPIRATORY DISEASE: Deficiency of vitamin A is known to have multiple effects on the epithelium of the respiratory system. In particular, the morphology of the trachea shows striking stratified squamous keratinised metaplasia; a characteristic seen in COPD patients, replacing normal mucus-secreting epithelia of the bronchi tree and also displays a destructive manner of alveolar structure. This effect was most noted in weaning rats fed on a diet devoid of vitamin A. This structural effect has profound results on premature human newborns that develop the respiratory condition known as bronchopulmonary dysplasia (BPD) (Zachman. 1989, Chytil. 1992, Shenai and Chytil. 1996, Chailley-Heu, Chelly et al. 1999). Many of the pathological features of BPD including atelectasis (the collapse of lungs), loss of ciliated cells, keratinising metaplasia and necrotising bronchiolitis, are similar to those pathological features found in patients with VAD (Chytil. 1992, Shenai and Chytil. 1994, Grummer and Zachman. 1995, Zachman and Grummer. 1998). In animal models, complete absence of vitamin A results in preterm spontaneous abortions, which in turn makes it impossible to study the full course of foetal development, however, animals that do survive are only under partial deficiency (Frey, Egli et al. 2004).

3.1.2: AIM. In this study, examined the morphological and morphometric effects of a diet deficient of Vitamin A in postnatal rat lungs from 6months up to 1 year. Vitamin A is essential is involved in the role of growth, development and structure of the lung. Chronic deficiency of this vitamin at a critical stage of postnatal growth in lung development results in gross abnormalities creating emphysematous-like features. We first looked at lungs of animals fed on this diet for 6 months. Data showed that there were some initial changes in the development of these lungs, with

the beginnings of an increase in alveolar airspace, vascularisation and alveolar septal damage. However, after examining rat lungs for a further 6 months (i.e. 1 year), we showed a longer period of this diet increased morphological features seen in 6 months lungs, but more pronounced, identifying that vitamin A is very much an important aspect of structure and functioning of the lung postnatally.

3.1.3: RESULTS: MORPHOLOGICAL AND MORPHOMETRIC ANALYSIS

MORPHOLOGY: Control rats were treated with PBS and peanut oil/DMSO (Dimethyl Sulfoxide). They displayed lung morphology and airspace (L_m) measurements to be distinguishable from normal, untreated lungs; confirming that this animal group had no structural effect and could therefore be used as controls.

Representative histological images of control and VAD animal groups of 6 months are shown in figure 3.1.1 (Fig 3.1.1a and b, scale bar: 100µm). VAD lung groups displayed subtle morphological changes when compared with 6 months control lungs. VAD lungs showed the beginnings of alveolar expansion accompanied by structural changes. This group also showed an increase in vascularisation. Capillaries were amplified within alveoli cell wall thinning (Fig 3.1.1b). Animals fed on a VAD diet for up to 1 year showed an increase in morphological changes (Fig 3.1.3d). The enlargement in 1 year VAD lungs had markedly increased from airway space in 6 months VAD lungs with extensive thinning of alveoli cell walls. There was also septal shrinkage; were more ‘pointed’ in this group (Fig 3.1.1d). Overall appearance of 1 year VAD rat lungs showed a resemblance to emphysematous lung structure. This appearance was mostly diffused throughout the lung. The

morphology of its counterpart showed typical alveolar airspace structure seen in normal healthy lungs.

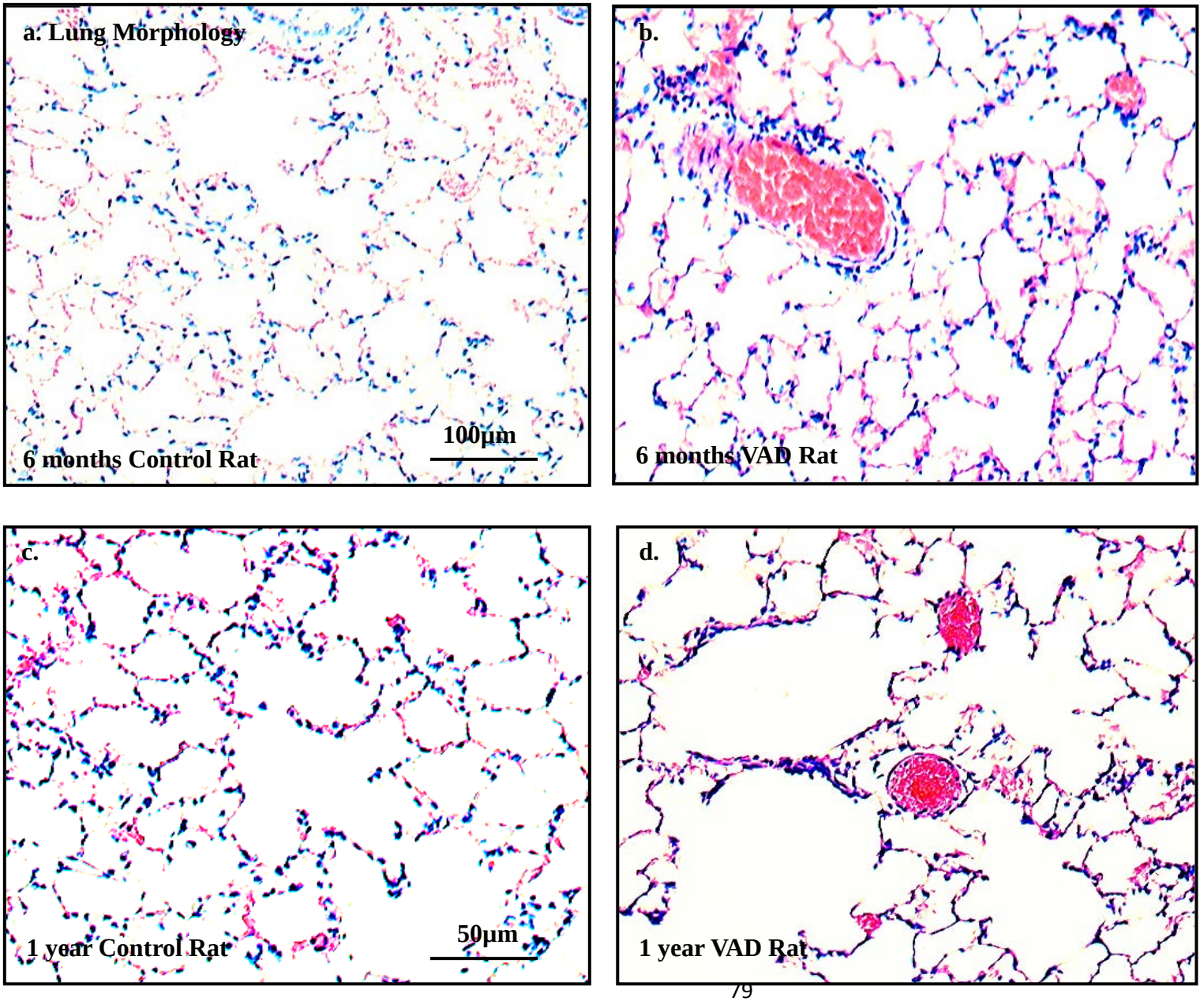

Fig 3.1.1 Lung Morphology. Figure shows representative transverse lung sections of 6 months and 1 year VAD animals (H&E staining a-d). Sections show typical structure of alveoli and vascularisation seen in postnatal rats of 6 months and 1 year on a diet deficient of vitamin A compared with control rats. There is a gradual increase in alveolar airway space and vascularisation between 6 months and 1 year VAD rat lung. Scale bar a-b: 100µm, c-d: 50µm

MORPHOMETRICS ANALYSIS (MEAN AIRSPACE CHORD LENGTH: Lm): Morphological changes observed from H&E stained sections were used for the quantification of airspace using the mean chord airspace chord length (L_m) measurement. Calculated L_m measurements were expressed in micrometres (µm). Data obtained from 6 months and 1 year VAD animals indicated a gradual increase in alveolar airspace. The mean value of airspace in 6 months VAD lungs was 102 ± 2.4µm this value increased to 135 ± 5.0µm in 1 year VAD lungs (Fig 3.1.2 and Fig 3.1.3, Table 3.1.1). The significance between these lung groups was <0.05 (p=0.007). These calculations along with morphology examination confirmed that dietary VAD had a defective affect in structural and hence functional lung activity. L_m calculations for 6 months and 1 year control lungs gave values of 84.3 ± 7.2µm and 92.8 ± 4.1µm, respectively (Table 3.1.1). These lower measurements taken from control groups not only indicated normal alveoli structure, but also suggested normal lung function and maintenance. The results suggest the alveolar area changing is due to alveolar expansion in size as Lm increases in dietary VAD for up to 1 year.

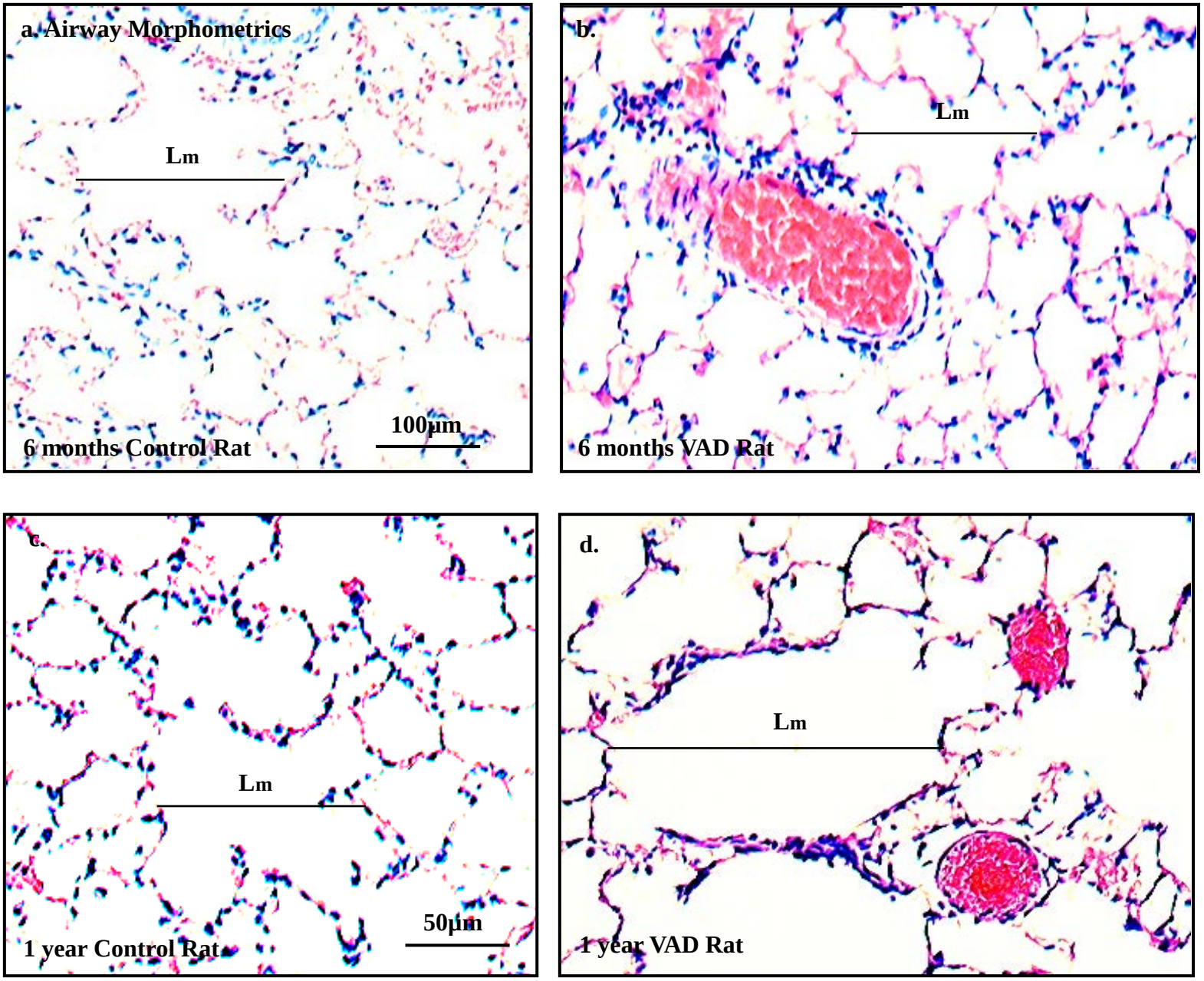

Fig 3.1.2: Morphometrics: Mean Alveolar Chord Length (Lm). Figure 3.1.4 shows representative H&E sections demonstrating airway measurements taken from 6 months and 1 year postnatal rat lungs. Fig b and d show chronic effect of VAD diet on alveoli airspace. The black lines indicate what parameters are measured. Scale bar: A and B= 100µm, C-D = 50µm

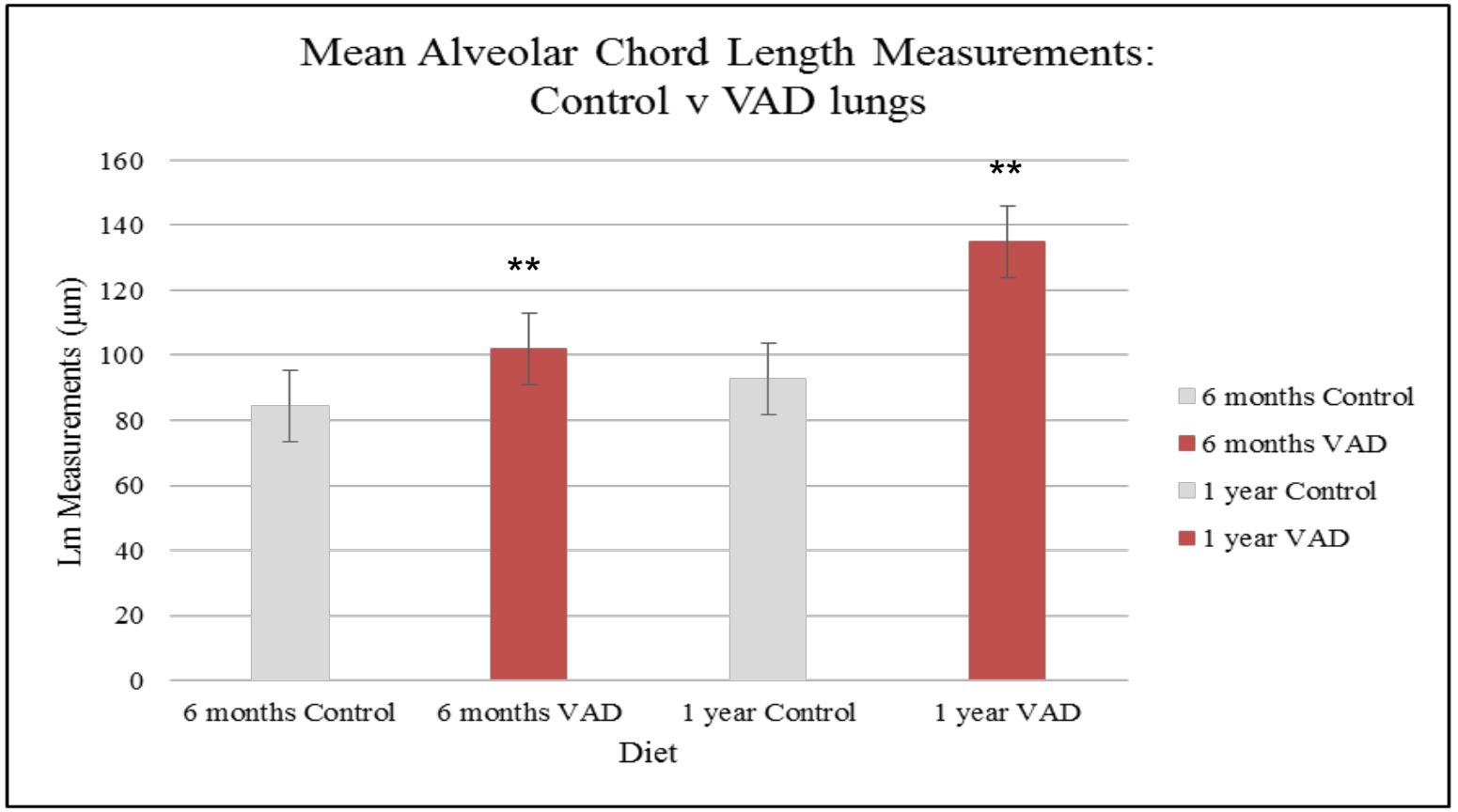

Fig 3.1.3: Mean (SEM ±) Chord Alveolar Airspace (Lm). Graph shows confirmation of the increase of alveolar airway space in VAD lungs from 6 months to 1 year. N = 5 per group. ** P<0.01 (Student unpaired t-test)

Table 3.1.1: Morphometric parameters of the dietary effect of VAD on Alveolar airway space in 6 months and 1 year Rat lungs

	6 month Control	**6 month VAD**	**1 year Control**	**1 year VAD**
Total Lm^2 (µm)	421.45	510	493.75	675
Sa 100g (cm^3)/10g	43.1	31.1	22.0	31.1
Mean	84.3	102	92.8	135
SD	1.9	5.3	5.3	4.8
SEM	5.4 ± 7.4	11.6 ± 7.0	5.55 ± 0.7	14.6 ± 5.6

Student unpaired t-test. Data is presented as ± SEM

THICKNESS OF CELL WALL: Alveolar cell wall of 6 months VAD lungs appeared thick compared to cell walls of 1 year VAD lungs (Fig 3.1.4b and d). 1 year VAD cells wall thickness showed resemblance to cell wall thickness clinically seen in emphysematous lungs. This observation could suggest that in order for dietary VAD to have an effect on alveolar cell wall, the diet needs to be for a period longer than 6 months.

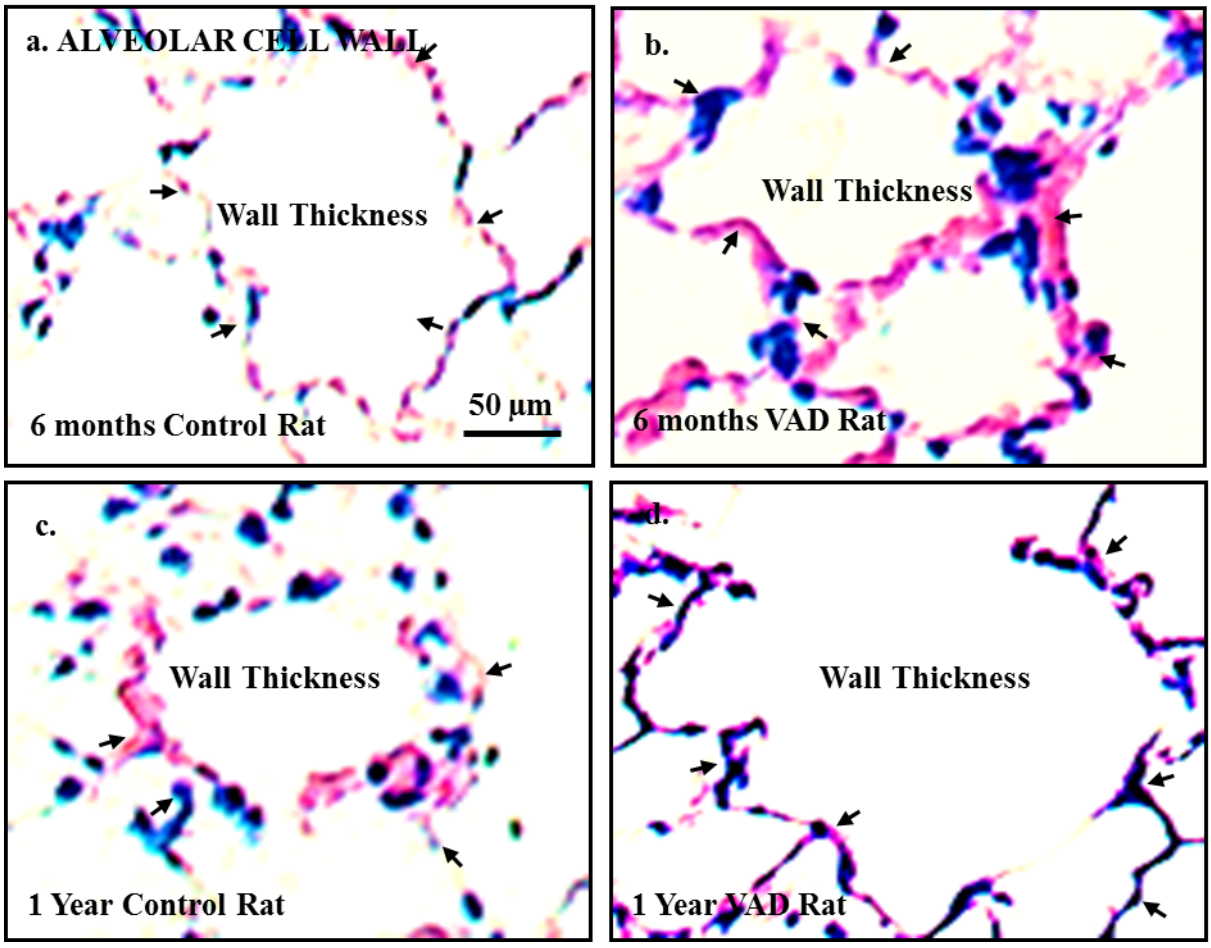

Fig 3.1.4: Alveolar Cell Wall Thickness. Representative pictures of alveolar cell wall thickness in 6 months and 1 year VAD rat lungs. There is a distinct difference in wall thickness between the two groups.

ALVEOLAR SEPTA: Observations of alveolar septa gave interesting findings. In 1 year VAD lungs, septa seemed thin with pointed ends. Some appeared with what looked like ‘chopped’ ends compared with three remaining groups. The septa in these lungs appeared thicker with rounded ends (Fig 3.1.5a-d). Septa in 1 year VAD lungs appeared much further away from neighbouring septa than those seen in the other groups. The increase in pulmonary vascular pressure may have been augmented by the destruction of the pulmonary capillary bed due to the destruction of alveolar septa seen in 1 year VAD lung group.

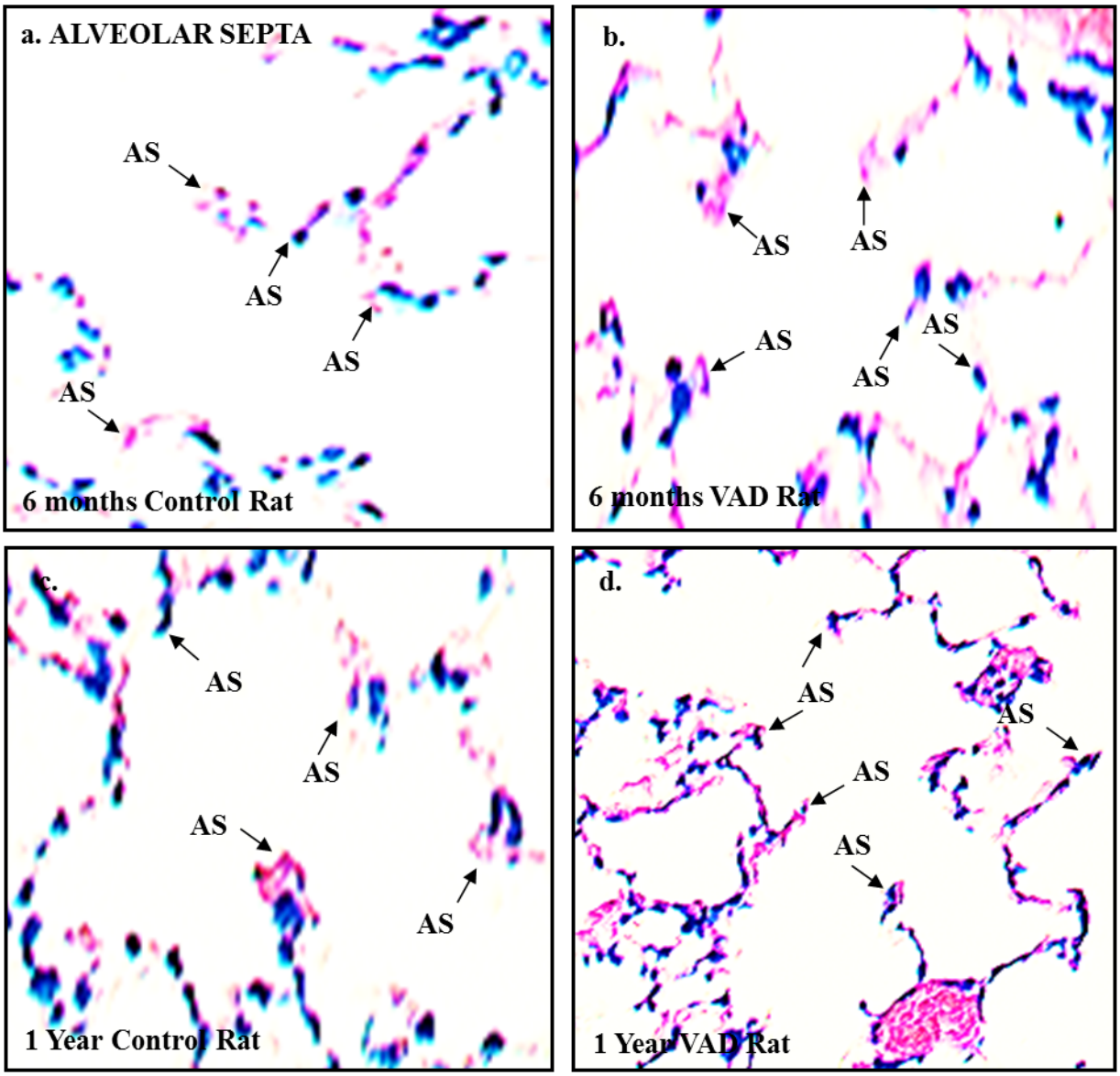

Fig 3.1.5: Representation of alveolar septa (AS). 6 months control (a), 6 months VAD (b) and 1 year control (c) lungs displayed thick rounded septa. 1 year VAD lungs (d) displayed thin and in some areas, pointed septa.

3.1.4: DISCUSSION: Starvation has long been known to induce histological appearances of emphysematous lungs and was noted by physicians performing post mortem examinations in Warsaw ghettos (Winick 1979). This phenomenon has also been studied in animal models of emphysema (Harkema, Mauderly et al. 1984, Kerr,

Riley et al. 1985, Karlinsky, Goldstein et al. 1986). Molecular mechanism(s) behind starvation-induced emphysema suggest that it is calorie-related and that alveolar destruction and regeneration occurring within a rapid timescale appear to be vitamin A (retinoid) – independent (Massaro, Radaeva et al. 2002). In previous studies, severe VAD during gestation results in multiple malformations in the foetus with foetal growth retardation or death (Frey, Egli et al. 2004) . In these studies, liver stores of vitamin A were completely drained by placing rats for 2 or 3 months (before mating) on a diet containing enough carotene for growth, but not for the storage of vitamin A (7-8 weeks are requires to deplete vitamin A from the liver) (Duncan, Green et al. 1993, Frey, Egli et al. 2004). These animals were transferred onto a diet completely free of carotene and vitamin A which was maintained throughout the pregnancy (Frey, Egli et al. 2004). Circulating vitamin in the mother and in the foetus became undetectable within a few days and resulted in severe developmental defects (Frey, Egli et al. 2004). By contrast, the aim of our experiment was to achieve VAD diet in postnatal rats and determine whether deformities, characteristic of emphysematous lungs were accomplished.

To examine the structural effect VAD diet had on postnatal rat lungs, we developed a cohort of VAD rats over a 1 year period. As previously described (Chapter 2.1), these rats were maintained on a retinoid (vitamin A) free diet from 12 weeks of age to 1 year after which they were sacrificed (Burri 1974, Hind and Maden 2004).

MORPHOLOGICAL AND MORPHOMETRICAL CHANGES: Light microscopy examination showed visible morphological changes in animals on deficient vitamin A from 6 months to 1 year. There were some obvious differences between the two groups, for example, the expansion of alveolar airway space. There was an exponential increase in Lm measurements from 6 months to 1 year animals, with an increase in vascularisation. Structural changes observed in 1 year VAD rat lungs resembled were characteristic of that seen in emphysematous lungs of patients with the disease confirming vitamin A importance in lung growth, structure and function. The increase in surface area is likely due to an increase in the complexity of vascularisation in VAD lungs and may be due to septal destruction in response to injury. There were clear differences between 1 year VAD and 1 year control lungs. VAD lungs appeared to have fewer, larger airspaces; alveolar cell walls were thinner with alveolar breaks and their septae were largely pointed and further away from neighbouring septae. There was a greater increase in vascularisation in these lungs than witnessed in 6 month lungs, suggesting greater activity of the repair mechanism taking place due to chronic tissue damage (Fig 3.1.3c and 3.1.4c and d). This effect was observed throughout these lungs. As no functional tests were carried out on these animals, we could only speculate that morphological features observed in 1 year VAD animals were not ideal conditions for respiration and that this structural abnormality may easily form chronic lung diseases such as emphysema and BPD. To help assess this assumption, we quantified the affected airspace size of each animal group (6 month control with VAD and 1 year control with 1 year VAD), by the use of morphometric analysis. Morphometric analysis confirmed morphological observations between 6 months and 1 year VAD rat lungs. Average mean chord length measurements showed a definitive increase in alveolar airspace from 6

months (102 ± 2.4μm) to 1 year (135 ± 5.0) lungs. The control counterparts for each diet gave averages of 84.3 ± 7.2μm and 92.8 ±4.1μm. When calculating the average differences between control and VAD lungs, the data showed twice the expansion rate of Lm from 6 months VAD (9.4%) to 1 year (18.6%) VAD lungs. This increase in Lm was significant and demonstrated that prolonged deficiency of vitamin A cause detrimental damage to lung structure.

Overall changes examined from morphology in VAD animals were increase in Lm, lug volume and surface area. These findings may reflect on a physiological compensation to preserve surface area that is similar to lung volume changes which occur in patients with emphysema. It is difficult to conclude whether VAD restricted diet is related to emphysema, especially as these rats used in this experiment had a nutritionally complete diet apart from the lack of retinoids, but experiments of similar design indicate that this may be the case in this model. For example, in an early VAD study by Sahebjami H and Vassallo CL (1979), a significant increase in Lm values was reported. Subsequent studies by Harkema JR et al (1984), Karlinsky JB et al (1986) and Sahebjami H and MaeGee J (1985) gave similar results and supports our 1 year VAD rat model.

Although the role that vitamin A plays in the development and pathogenesis of COPD/emphysema is not well understood, data from animal experiments seen in models such as elastase-induced emphysema, suggest that vitamin A supplementation may help prevent adverse respiratory outcomes caused by emphysema. Data provided from our model and other models based on VAD diet

and caloric deficient diets, provide the possibility that vitamin A causes adverse structural abnormalities in postnatal lungs, which in effect could alter their respiratory function. The VAD diet results in grossly enlarged alveolar airway surfaces, extensive thinning of alveolar cells walls with distorted features of alveolar septae synonymous with pathological features of emphysema. This effect can be seen in a diet prolonged deficiency of vitamin A in premature infants as well as infants in poor environmental conditions, where its absence disrupts the performance of respiration.

3.1.5: SUMMARY. VAD rat lungs shows severe caloric restriction lead to alveolar enlargement or emphysema. The effects of postnatal VAD may be species-dependent; although starvation of hamsters caused an enlargement of airspace and a reduction in surface area, but it was not associated with a reduction of pulmonary content or evidence of alveolar wall destruction. Such differences may be due to differences in somatic growth at the time of the nutritional insult. In general, however, it appears that restricted nutrition leads to protein degradation, impaired cross-linking of pulmonary structural proteins and an imbalance between synthetic and catabolic processes, all of which may contribute to the remodelling that results in emphysematous changes in postnatal lungs.

CHAPTER 3.2

THE EFFECT OF DEFICIENT VITAMIN A ON PROGENITOR CELLS OF THE LUNG

OBJECTIVE: The overall study was to characterise changes of airway progenitor cells; alveolar type II (ATII) cells and Clara cells in the alveolar and tracheobronchial epithelium of 1 year postnatal VAD rat lungs. We evaluated both cell population, proliferation and transcriptional alteration of these progenitor cells. The data obtained was accomplished by the following techniques; immunohistochemistry and Immunofluorescent labelling along with cell counting from at least 5 non-overlapping microscopic fields (at x40) and immunoblots. Morphological analysis of CC10 positive Clara cells demonstrated in VAD bronchiole epithelium showed distinct structural change in the bronchiole epithelium from structures of CC10 positive Clara cells in control groups. Cells were less columnar in shape and their classical 'cap' situated on top of these cells were absent, but there appeared to be no real change in the number of cells expressed in either animal group. SP-C positive ATII cells located in alveolar epithelium were small and irregular in shape in VAD lungs. Staining of these cells in this group were spatial and cell numbers had significantly decreased in comparison with control lungs. SP-C positive ATII cells in control lungs were more oval in shape with clearly defined nuclear staining. Cellular expression was more compact. There was also a reduction in transcriptional and proliferative activity of both Clara and ATII cells in VAD lungs. **Conclusion:** VAD diet greatly affects the airway epithelium and is noted by the pathophysiological response of both Clara and ATII cells in the respiratory tract. Both transcription and proliferation of these cells are also affected which are cruel to the functioning and maintenance of the lung.

3.2.1: INTRODUCTION. Adequate dietary intake of vitamin A is essential for the up keep of the respiratory epithelium. Abnormalities exhibited in VAD rat lungs have been shown to have similar clinical features observed in premature infants with poor nutritional diet (McMenamy and Zachman 1993, Baybutt, Hu et al. 2000). Dietary studies have defined the role of vitamin A in tracheal epithelial cells after injury, suggesting that regeneration of the tracheal epithelium after injury requires vitamin A, but little research has looked into the effects of VAD in airway cells, in particularly progenitor cells (Klann and Marchok 1982, McDowell, Keenan et al. 1984, McDowell, Ben et al. 1987, Lancillotti, Darwiche et al. 1992, Baybutt, Hu et al. 2000). The development of advanced biochemical tools and techniques has been adapted over the years for the isolation of several airway cell types including ATII cells and nonciliated cells or Clara cells. From this development, over 40 varied cell types have been uncovered in the lung (Fig 3.2.1) (Devereux 1984).

Both ATII cells and Clara cells are unique to the lung and have been shown to secrete a protein known as surfactant which has two main functions; firstly to aid in the regeneration of surface tension (in the alveolar epithelium) and to act as part of the defence mechanism (in the bronchi and bronchiole epithelium). A major function of these cells is that both are progenitor cells are able to differentiate into specific cell types ATII cells for alveolar type I (ATI) cells and Clara cells for mucus secreting goblet cells; little else is known about these cell types.

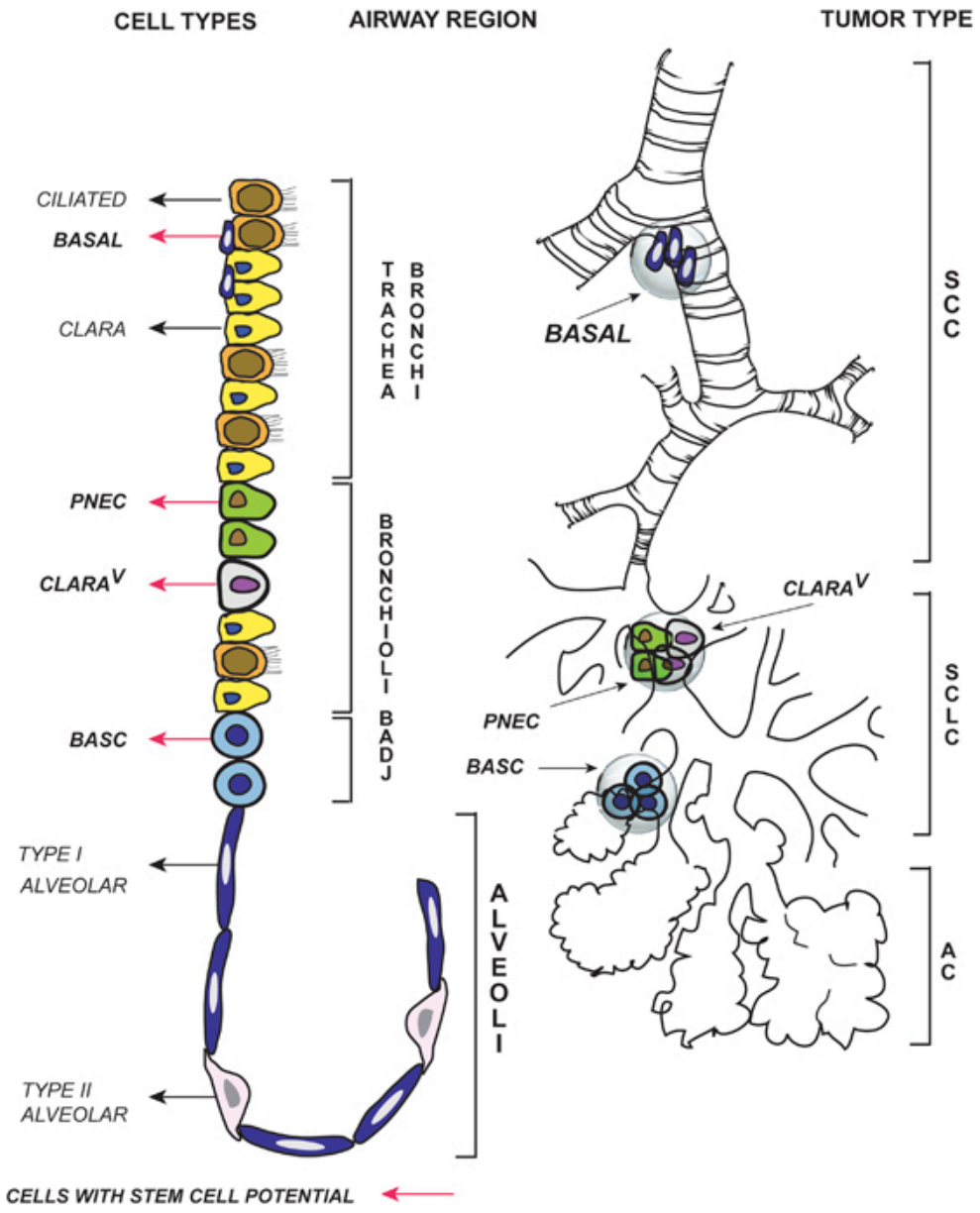

Fig 3.2.1: Airway progenitor cells. Schematic representation of the airways and types of cells present in each region. The red arrows indicate cell types with progenitor cell potential. *Eramo A et al. Lung Cancer stem cells: tools and targets to fight lung cancer. Oncogene.*

In the event that injury occurs, the epithelium is repaired by an abundant progenitor pool and a sequestered population of adult stem cells. Chronic lung diseases such as asthma, COPD and BPD are characterised by extensive epithelial remodelling resulting in a reduction to the number of both ATII and Clara cells, suggesting that epithelial repair has been compromised. In addition to effects on the epithelium, these diseases are also accompanied by extensive subepithelial fibroproliferation, mesenchymal remodelling and elevated extracellular matrix deposition as well as a profound increase to lung inflammation. The findings that airway epithelial cells are essential for modulation of innate immunity suggests that the enhanced inflammatory response described in chronic lung diseases may be a result of attenuated airway epithelial cell function.

Cell turnover of the epithelium lining the pulmonary airways is a slow process in normal lung maintenance. Methods used to calculate the rate of epithelial turnover rely on direct approaches that are based upon measurements of cell proliferation and lineage tagging. Measurements of cell proliferation have been principally replied upon stable incorporation of labelled DNA precursors such as bromodexyuridine (BrDU) or proliferating cell nuclear antigen (PCNA) into the DNA of cells traversing S-phase. Data generated from continuous labelling of a steady-state pulmonary epithelium, demonstrates the frequency of proliferation within the airways to be 1% per day (Hong, Berg et al. 2001). Assuming a direct relationship between the rate of epithelial cell loss and replacement, this data suggests that these cells have an average lifespan of 100 days.

Cell turnover in the lung epithelium is replaced far more slowly than the gut or the epidermis, a property that is reflected in the functional characteristics of airway progenitor cell in resting versus proliferative states. Although proliferation and differentiation of ATII cells is a critical factor in the repair of the pulmonary epithelium, regulation of the pulmonary healing processes is not well understood. It may well be that transcription factors play a key role in the control of tissue restoration after injury (Baybutt, Hu et al. 2000). However, there have been studies on cell growth stimulation and proliferation using cultured tracheal cells with the stimulatory agent being RA (Klann and Marchok 1982, Lancillotti, Darwiche et al. 1992, Baybutt, Hu et al. 2000). Less information is available concerning the role of vitamin A of the distal epithelial cells of the alveoli (Baybutt, Hu et al. 2000).

Increased risk of respiratory infections is partly due to squamous metaplastic changes in the epithelia of the respiratory tract seen in human VAD infants (Sommer 1983). Experimental induced injuries such as Naphthalene exposure has been documented in reducing Clara cell population and activating putative bronchiolar tissue-specific progenitor cells located within the neuroepithelial bodies (NEB) and bronchiolar-alveolar duct junction (BADJ) (Stripp 2008).

Nkx2.1 is a 43kDa phosphorylated member of the homeodomain-containing proteins expressed selectively in the lung, thyroid and central nervous system (DeFelice, Silberschmidt et al. 2003). Nkx2.1 is embryonically found in ATII and Clara cells and is therefore an important role in lung formation and function, but its involvement in the phosphorylation and transcriptional activation of target genes and

in cell differentiation in target tissues is unclear (DeFelice, Silberschmidt et al. 2003). Furthermore, Nkx2.1 expression is spatially regulated during lung morphogenesis, being highly expressed in peripheral regions during the process of lung budding with advancing development (Lazzaro, Price et al. 1991, Zhou, Lim et al. 1996, DeFelice, Silberschmidt et al. 2003). Because of its role in lung development, a large presence of this protein in treated postnatal lungs after the process of injury would suggest some form of lung repair/regeneration.

3.2.2: AIM: Chronic airway diseases are becoming of the leading causes of morality worldwide. As previously mentioned, there are very few effective treatments available to patients especially with COPD/emphysema to reverse or cure symptoms. Therapies using stem cells to regenerate lung tissue is fast growing and there is also the prospect of using progenitor cells as a therapeutic measure. The aim of this study was to first determine both Clara and ATII cell's ability to respond to airway injury initiated by a VAD diet and to evaluate the impact of VAD on cell turnover and differentiation of Clara and ATII cells.

3.2.3: RESULTS.

CC10 Protein Expression of Clara cells: On examination of 5µm sectioned lung tissue, light microscopy of DAB detected staining revealed CC10-positive Clara cells to be localised along the tracheobronchial epithelium in VAD and control rat lungs. We focused on CC10-positive Clara cells located in the bronchiole epithelium (Fig 3.2.2a and b). In control lungs, CC10-positive Clara cells presented a classical columnar epithelium dome-shape (Fig 3.2.2a). Immunofluorescent labelling of these cells revealed in more detail these cells to contain an apical 'cap' and there was an evenly, uniform distribution of these cells (Fig 3.2.2a and c). In VAD lung, these structures appeared different in the bronchial epithelium. They appeared 'plump' in size, they did not seem to have apical cap appendages and distribution was intermittent (Fig 3.2.2b and d). Along the bronchioles of VAD lungs lay pockets of grouped Clara cells. This feature was at times intermittent. Quantitative analysis of cell counts showed a small difference between the two groups. Statistically, Clara cell counts gave an average difference of 203.6 ± 11.0 in VAD lungs compared with control lungs which gave an average Clara cell counts of 248.2 ± 11.8. This produced a P value of 0.02 (Fig 3.2.3, Table 3.2.1).

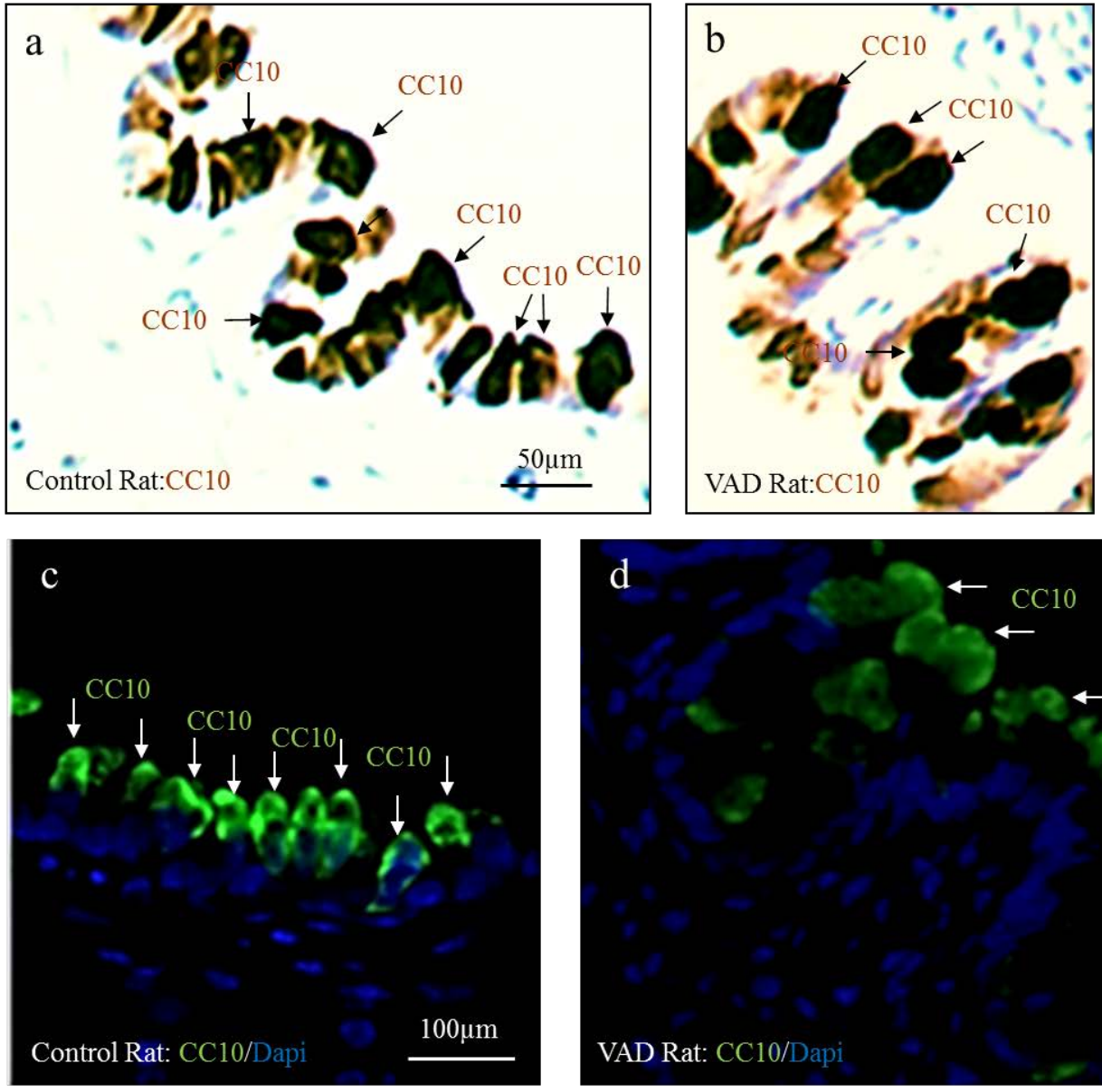

Fig 3.2.2: IHC and IF labelling of goat-polyclonal anti-CC10 positive Clara cells located in the bronchiole epithelium of control (a and c) and VAD (b and d) animal groups. Note: expression of Clara cells in VAD lungs appear less columnar and more plump than cells in control lungs, where they show classical Clara cell morphology. Scale bars = 50-100µm

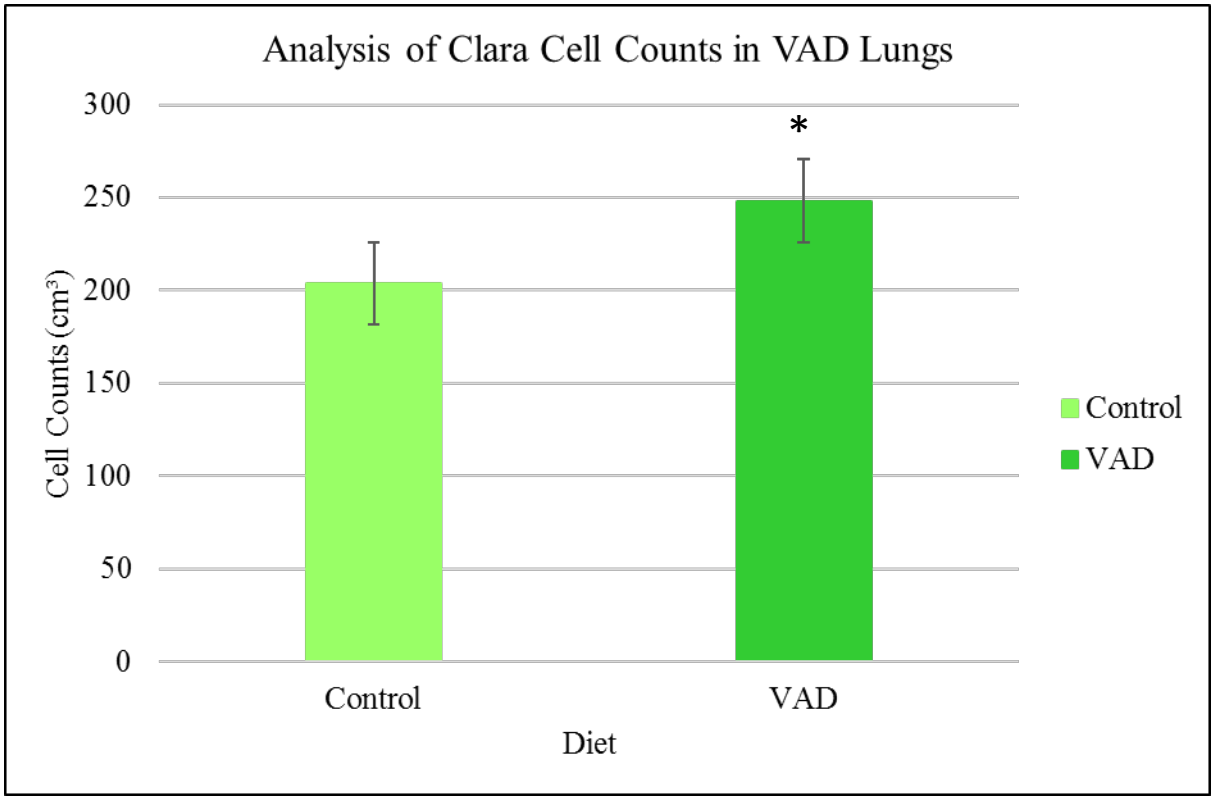

Fig 3.2.3: Graph displaying average cell counts of Clara cells in VAD lungs in comparison with control lungs. Cell counts were calculated using students t-test. The difference between the two groups was $P<0.02$

SPC Protein Expression of ATII cells: Next we evaluated cell population and distribution of SP-C positive ATII cells located in the alveolar airway epithelium. ATII cells were evaluated using a rabbit polyclonal anti-SP-C antibody. IHC and IF staining of this antibody revealed secretory ATII cells in populated groups throughout the alveolar epithelium in control lungs (Fig 3.2.4a and c). There was positive SP-C cells situated at the ends of exposed alveolar septae; probably where budding was taking place. IF labelled cells made it more apparent that the staining pattern of these cells were prominent around the nuclei and that each cell was closely situated to one another (Fig 3.2.4c). Examining SP-C positive ATII cells in

VAD lungs revealed a number of changes. Firstly, the number of positive cells was much reduced from 59.6% in control lungs to 40.4% in VAD lungs. Secondly, ATII cells in VAD lungs looked as if shrinkage had occurred (Fig 3.2.4d). IHC stained section for SP-C showed that cells that were positive in both animal lung groups appeared granulated (Fig 3.2.4a and b). This observation suggest that these cells were undergoing cell division and perhaps undergoing differentiation into ATI cells, as it has been documented in the literature that when injury has taken place, ATII cells not only proliferate to make more ATII cells, but differentiate to create an ATI population as these cells do not have the capacity to proliferate into new ATI cells. Furthermore, IHC staining in VAD lungs showed some unusually large cells which surrounds populated groups of ATII cells. These cells looked like they may be macrophages and were frequent throughout these lungs (Fig 3.2.4b). Statistical analysis confirmed the number of positive ATII cells detected by anti-SP-C (Fig 3.2.5, Table 3.2.1). Overall, VAD lungs gave an average cell count of 156.8 ± 6, compared with control lungs whose average cell count was 231.0 ± 31.

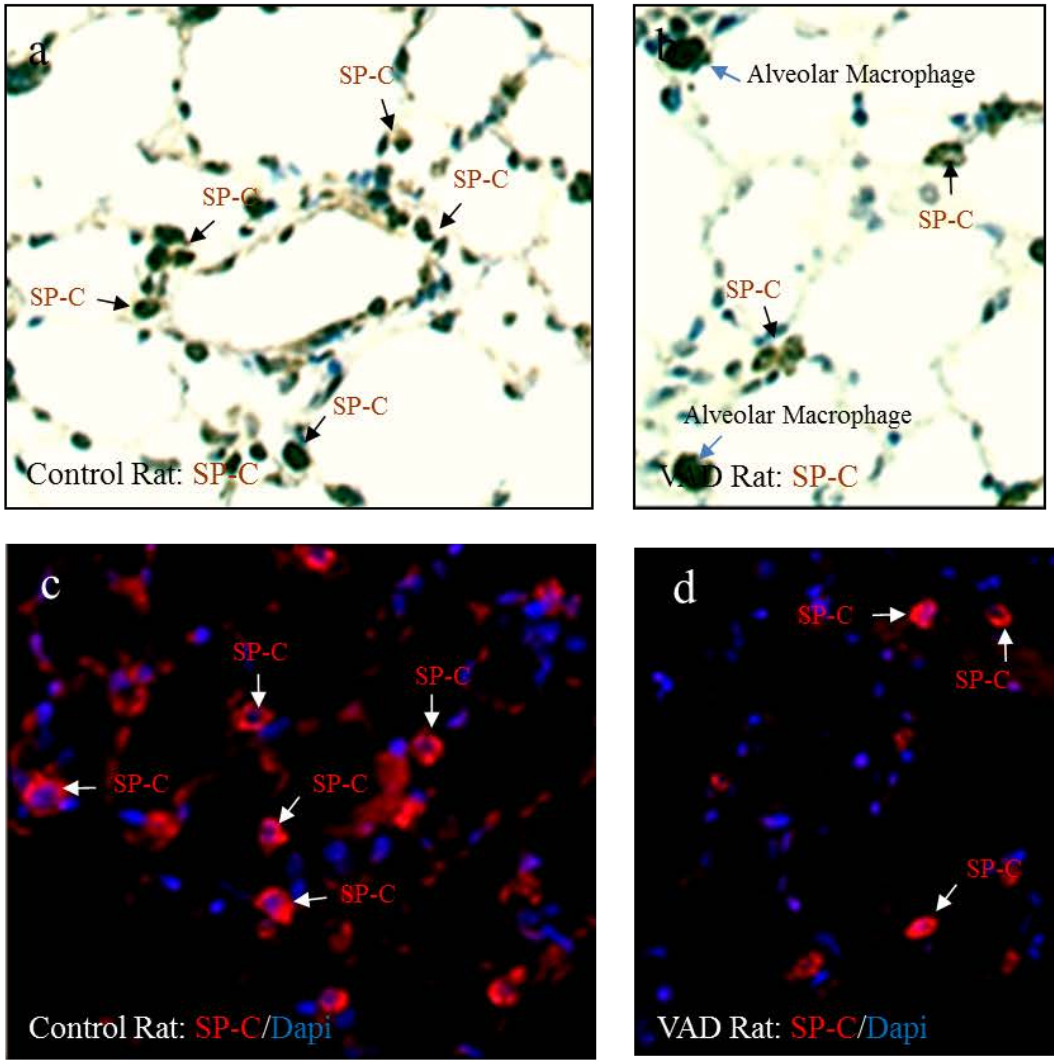

Fig 3.2.4: IHC and IF labelling of rabbit-polyclonal anti-SP-C positive C ATII cells located in the alveolar epithelium of control (a and c) and VAD (b and D) animal groups. There appears to be less SP-C positive ATII expression in VAD lungs than control lungs. There is the presence of alveolar macrophages (blue arrow). Scale bars = 50-100µm

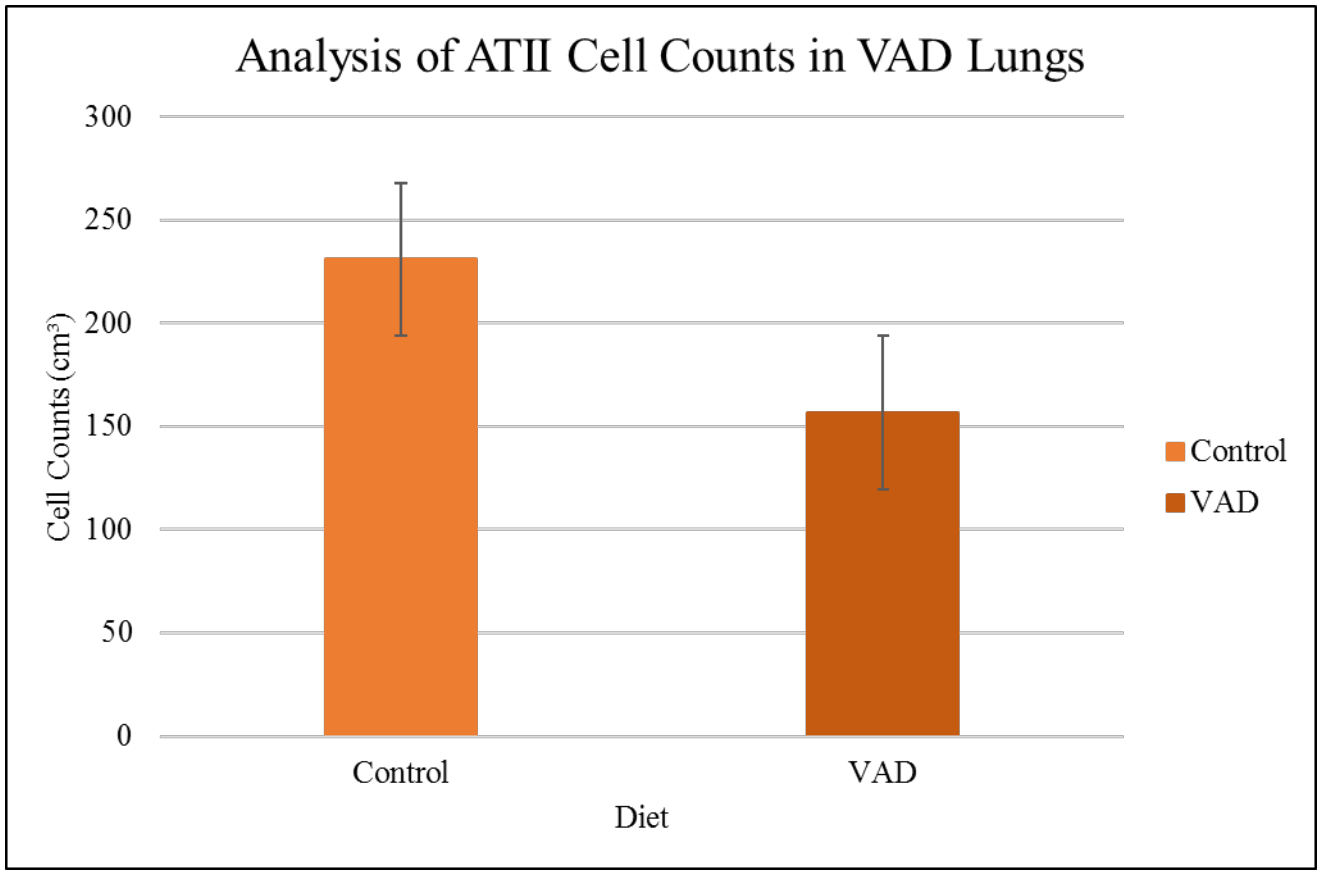

Fig 3.2.5: Graph showing the average number of ATII positive cells in the alveolar airway epithelium of VAD lungs in comparison to control lungs detected by rabbit anti-SP-C. The graph depicts a reduced number of ATII cells in VAD alveolar airway epithelium when compared with control lungs. Reduction of these cells could suggest cell differentiation into ATI cells.

Table 3.2.1: Average cell counts of CC10-positive Clara cells and SP-C positive ATII cells in the bronchiole and alveolar airway epithelium of VAD lungs compared with control lungs

Primary Antibody	Control Lungs	VAD Lungs
CC10: Clara cells	203.6 ± 11.0	248.2 ± 11.2
SP-C: ATII cells	231.0 ± 31	156.8 ± 6

Table shows that VAD has a significant effect on ATII cell population, whereas with Clara cell population the effect of VAD is minimal.

Nkx2.1 Protein Expression: We examined whether the presence of Nkx2.1, if any in postnatal lungs was altered in VAD lungs. Detection of Nkx2.1 was demonstrated using a mouse monoclonal anti-TTF-1 antibody. Nuclear localization of TTF-1 positive Nkx2.1 was clearly demonstrated in alveolar and bronchiole airway epithelium in control lungs. Cell population of TTF-1 Nkx2.1 positivity was seen in large numbers and in some areas, appeared grouped in alveolar epithelium (Fig 3.2.6a). Cell positivity for Nkx2.1 in VAD alveolar epithelium showed alteration in expression. Cells were darker and appeared larger and flatter and much harder to distinguish. Positive Nkx2.1 cells for this antibody were fewer and spacious (Fig 3.2.6b). Cellular expression was not present around alveolar septae in VAD lungs; this lack of expression was not seen in control lungs. Cell counts confirmed the reduction in the number of TTF-1 positive Nkx2.1 in VAD alveolar epithelium. The average count for this group was 27.2 ± 3 compared with cell counts in control

alveolar epithelium, where the average was 64.4 ± 2. The P value for this was 0.01, which statistically was deemed significantly different (Fig 3.2.7, Table 3.2.2). In terms of protein reduction in VAD lungs, expression of Nkx2.1 in the bronchiole epithelium showed similar results observed in alveolar epithelium. Positive labelling in control groups were shown to surround columnar epithelial cells in the same area where CC10-positive Clara cells were shown to be located (Fig 3.2.5C and Fig 3.2.2a and c) and also where goblet cells are normally situated. Staining was clear and uniform. The only concern about the expression pattern of this protein is that its labelling appeared different from published data examining Nkx2.1 in human bronchioles. Expression of TTF-1 positive Nkx2.1 protein in VAD groups was sparse with extremely weak staining (Fig 3.2.6d). The average cell counts for these cells located in the bronchiole epithelium were as follows, 50.0 ± 2 in control lungs and 20.2 ± 2 in VAD lungs (Table 3.2.2). These values also gave a significant difference of 0.01 (Fig 3.2.7).

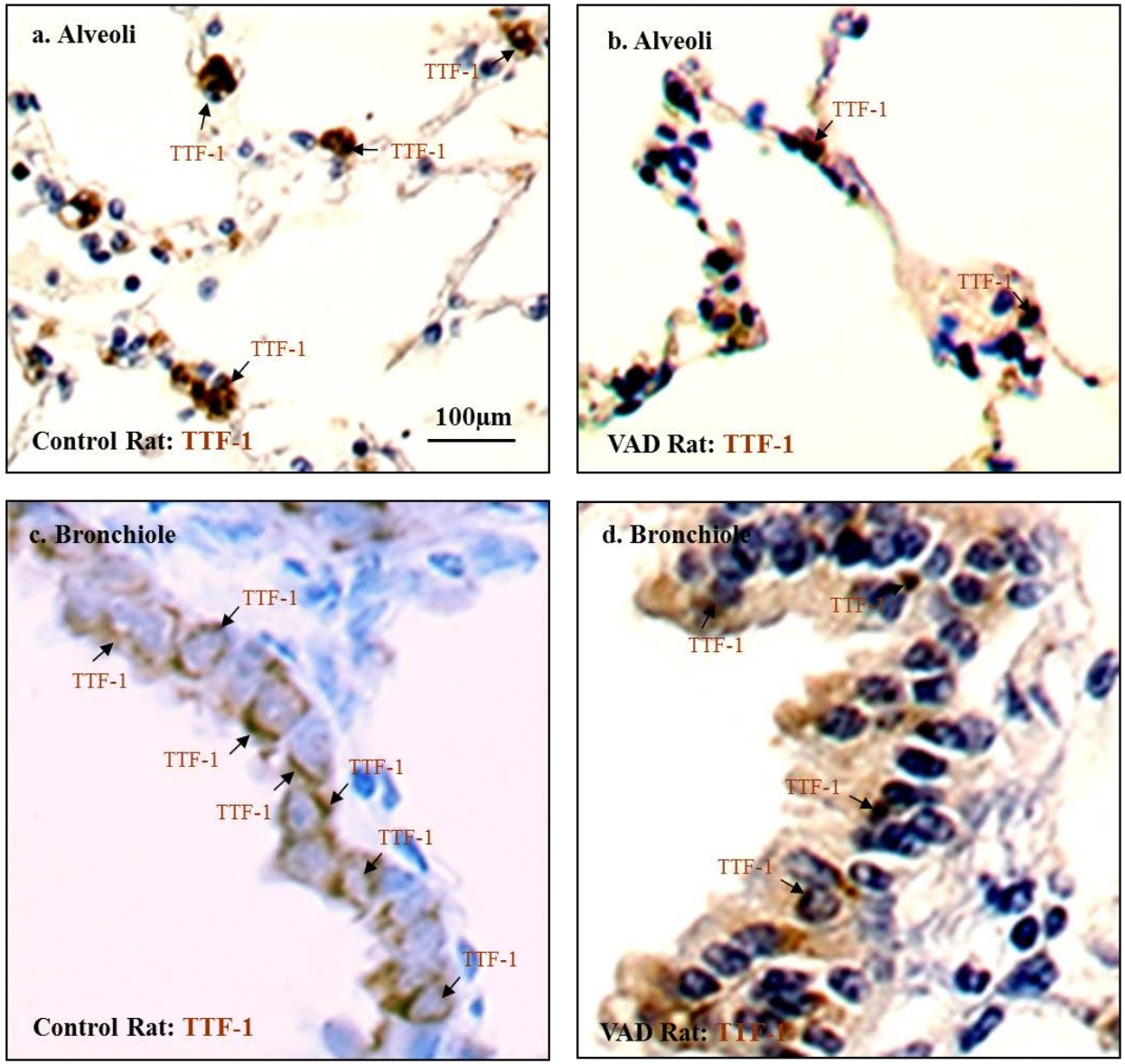

Fig 3.2.6: IHC labelling of TTF-1 positive Nkx2.1 in alveolar and bronchiole airway epithelium of control and VAD lung groups (b and d). Expression of this transcription factor was weakly positive in VAD lungs suggesting inhibition of transcription activity promoted by this diet. Scale bar: 100µm

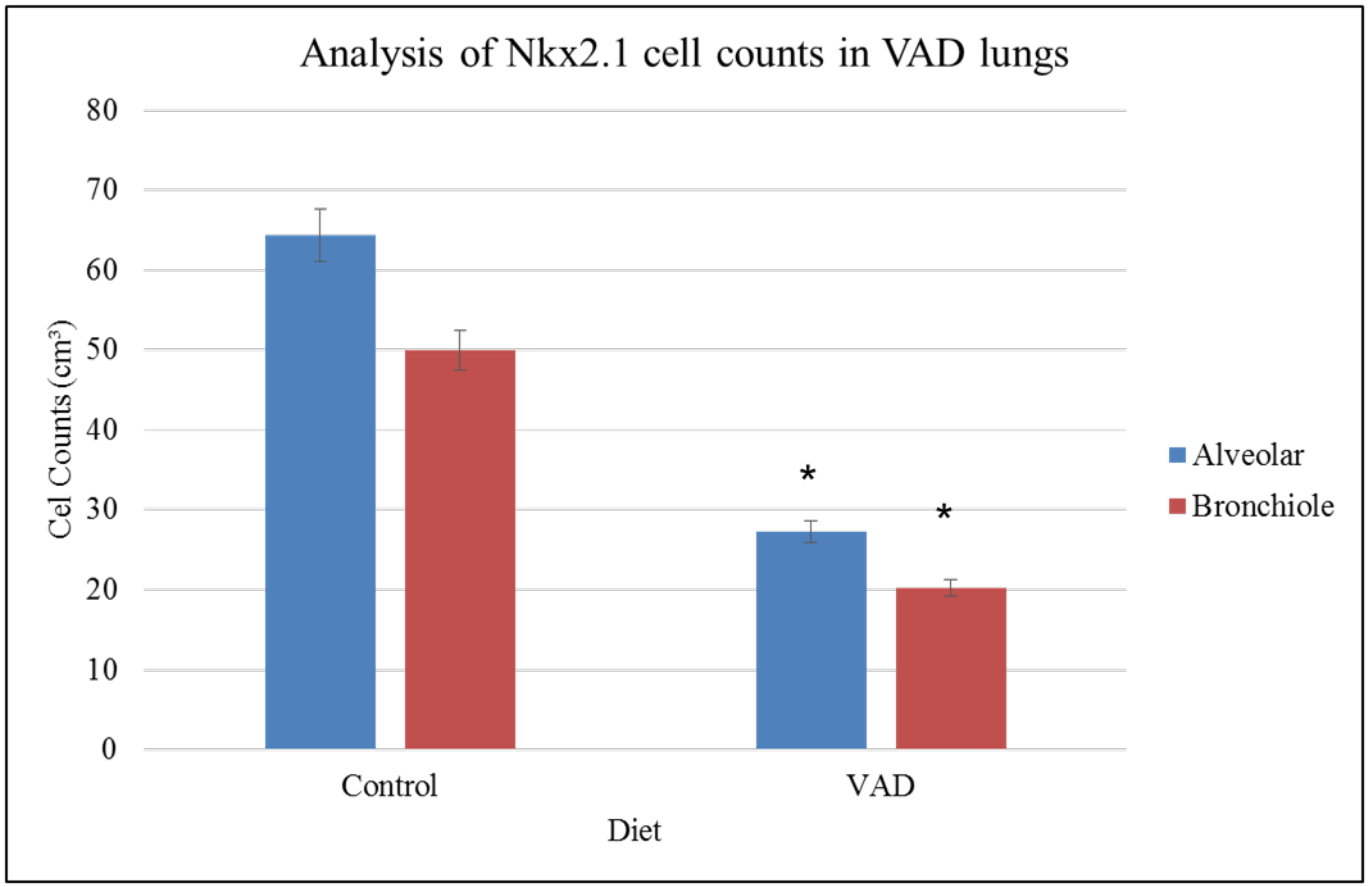

Fig 3.2.7: Average TTF-1 positive Nkx2.1 cell counts. Cell counts were calculated using student's t-test. TTF-1 positive Nkx2.1 cells were significantly lower in VAD postnatal rat lungs compared with control postnatal lungs. $P<0.01$.

Table 3.2.2: Analysis of Nkx2.1 transcriptional activity in postnatal VAD lungs

	Control Lungs	VAD Lungs
TTF-1 (Alveolar)	64.4 ± 2.0	27.2 ± 2.0
TTF-1 (Bronchiole)	50.0 ± 2	202 ± 2

Immunoblot Analysis of CC10, SPC and TTF-1 protein: Analysis of protein expression via immunoblotting using homogenates of both lung groups showed detection of CC10, SP-C and TTF-1 positivity (Fig 3.2.8). There were varying degrees of expression levels amongst these proteins. This set of results was in conjunction with IHC and IF labelling (Fig 3.2.2 -3.2.8). The reduction of SP-C and Nkx2.1 positive TTF-1 suggest that postnatally, SP-C is still regulated by Nkx2.1, but CC10 is not, as levels were unaffected by VAD.

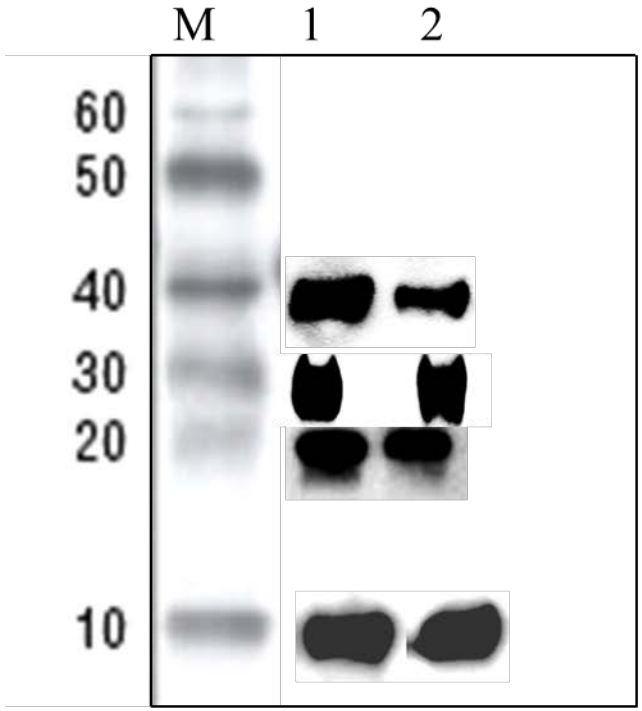

Fig 3.2.8: Immunoblot of airway progenitor cells and transcription factor. The effect of VAD diet on alveolar and bronchiole progenitor cells. CC10 and SPC protein were used to detect Clara and ATII cells of the bronchiole and alveolar epithelia, along with TTF-1 for NKx2.1 transcription factor. Equal volumes of control (Lane 1) and VAD (Lane 2) lung homogenate tissue (15µl) was loaded onto a 4-12% Bis Tris gel before being transferred and immunostained with the above named proteins. With the exception of C10 staining, immunoblots showed a reduction of SPC TTF-1 and PCNA expression.

Proliferation Analysis of Clara and ATII cells: Finally, we examined the proliferation of Clara and ATII cells in VAD lungs. We know from previous experimental studies that chronic lung injury causes the proliferation of cells to greatly alter. Proliferation of progenitor cells in healthy lungs is normally slow, but in abnormal circumstances, such as diseased or injured lungs, proliferation can be either seen to increase or decrease dramatically. IF labelling with the proliferative antibody, PCNA with CC10 and SP-C revealed positive proliferation of SP-C positive ATII cells in both VAD and control alveolar epithelia (Fig 3.2.9c and d). There was a reduced number of proliferative ATII cells in VAD lungs compared with control lungs. Calculated averages showed 44.2 ± 1 in VAD lungs and 72.4 ± 4. The reduction in the number of ATII cells in VAD lungs could be due to cell differentiation of the cell population to ATI cells as from morphological studies we deduced epithelial damage where both cells are located for the regulation of surface tension (ATII) and the process of gas exchange (ATI). Because we did not carry out double labelling of ATII and ATI cells, we can only hypothesize that this was the cause of ATII cell reduction in VAD lungs as it has been well documented that ATII cells not only proliferate into more ATII cells, but differentiate into ATI cells, as these cells do not have the capacity to do so themselves in a diseased or injured environment. Proliferation of Clara cells was only detected in very small numbers in VAD bronchioles and not in control bronchioles (Fig 3.2.9b). However, proliferation of basal cells were highly detected in control lungs (Fig 3.2.9a). Even though the two groups detected two sets of proliferative cells from the bronchiole, cell counts were still carried out. Basal cell counts in control lungs gave an average of 101.2 ± 2 whereas average cell counts of Clara cells in VAD lungs were 43.5 ± 5. (Fig 3.2.10).

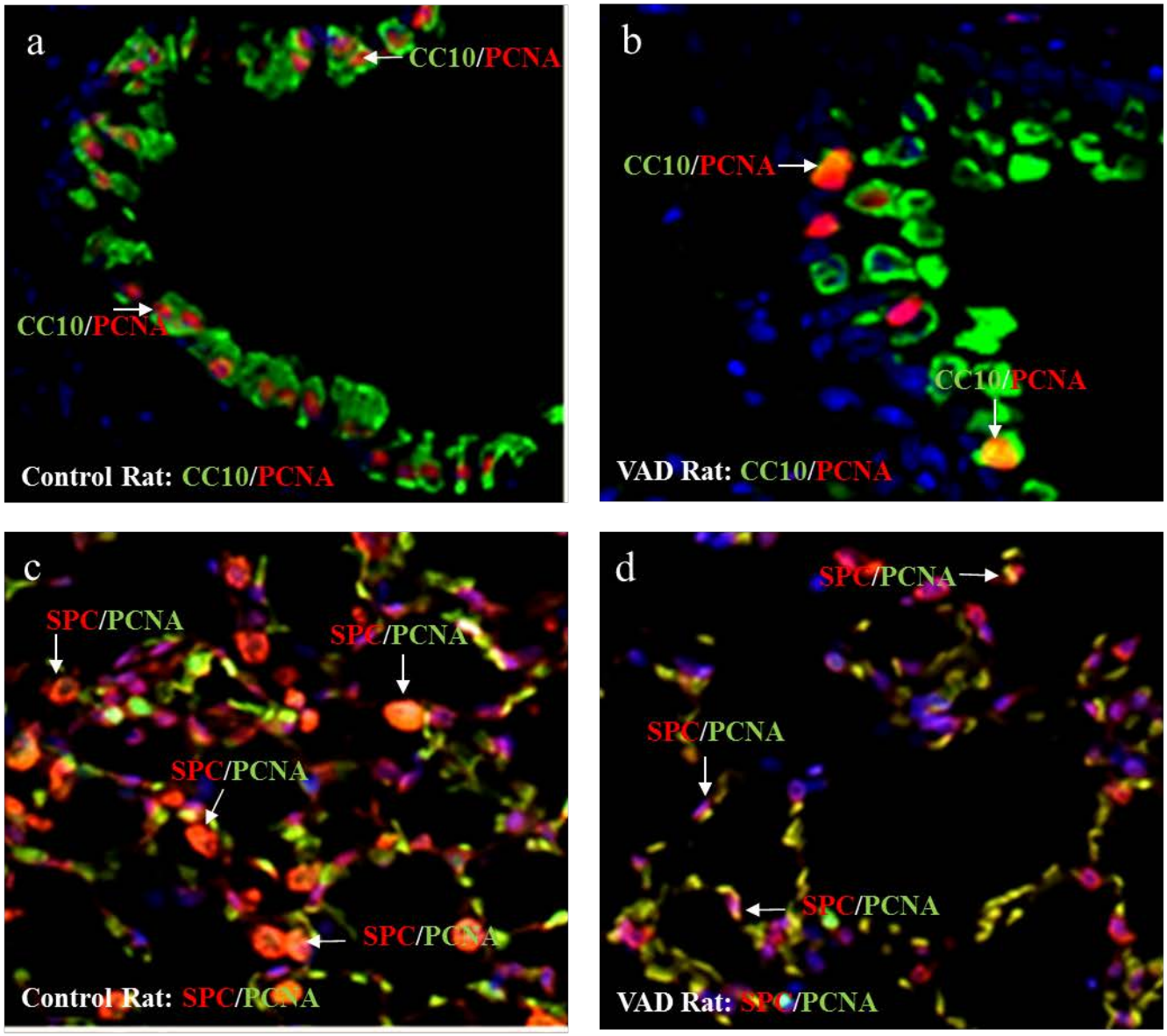

Fig 3.2.9: IF double-labelling of proliferative Clara (a and B) and ATII (c and d) cells. Figures b and d show very few proliferative activity of Clara and ATII cells in VAD lungs, whereas figures a and c show proliferative basal (a) and ATII cells in control lungs.

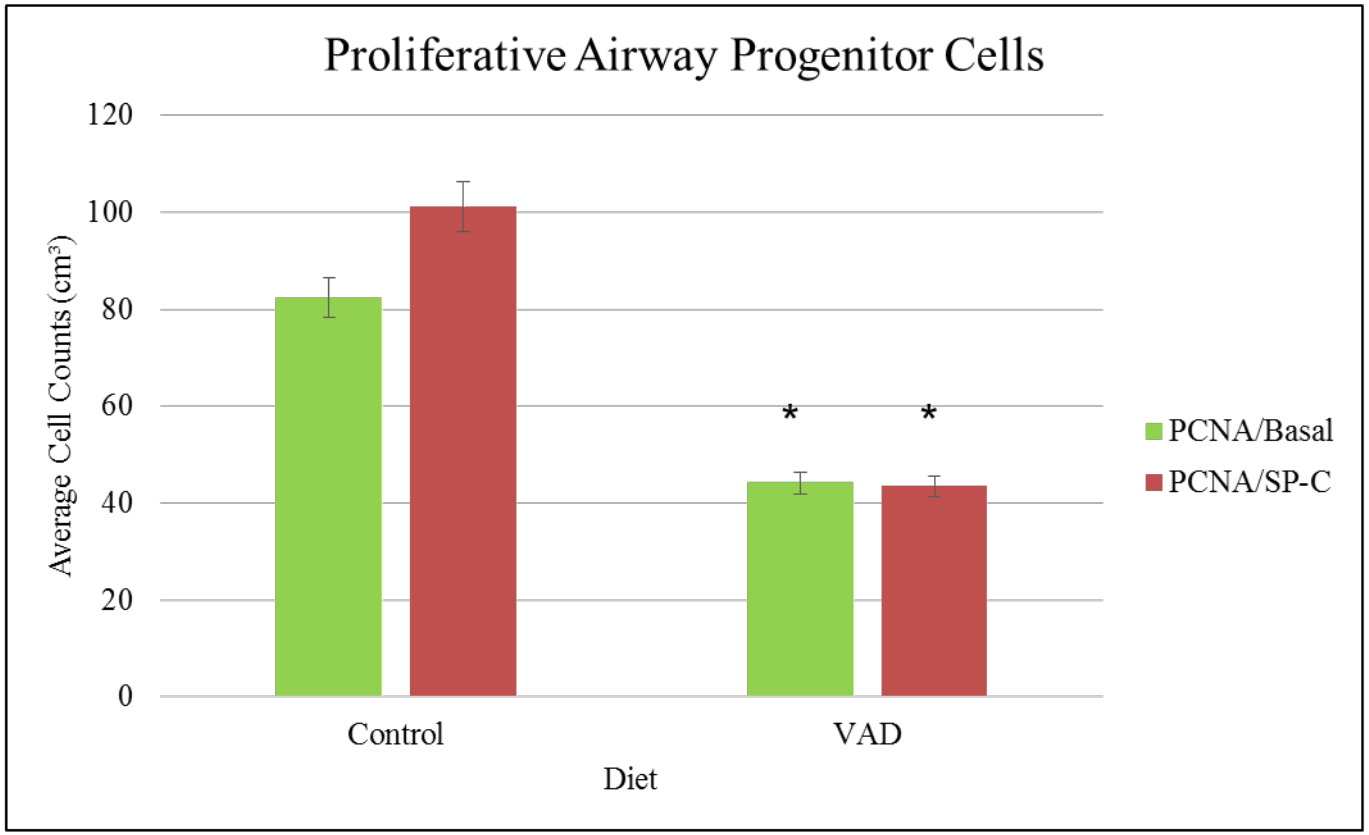

Fig 3.2.10: Average cell counts of proliferative airway progenitor cells in VAD and control lung groups. Basal cells – green bars, ATII cells - red bars. There was a clear reduction of proliferative basal and ATII cells in VAD lungs. Clara cells showed very little proliferative activity in both VAD and control lungs.

Immunoblots of PCNA revealed high levels of proliferation in the two groups. This high level of proliferative activity in both groups can be justified as both Clara and ATII cells were not isolated for this technique, so we can only surmise that high proliferative activity could be due to other cellular populations interacting with PCNA in the lung (Fig 3.2.11).

Table 3.2.3: Average proliferative cell counts of airway progenitor cells

Primary Antibody	Control Lungs	VAD Lungs
PCNA/CC10	82.4 ± 4	44.2 ± 1
PCNA/SP-C	101.2 ± 2	43.5 ± 5

3.2.4: DISCUSSION. Defective airway epithelial repair has been proposed as an early event in the initiation of chronic airway diseases. Chronic defects in reparative capacity and cellular composition may further contribute to disease progression and susceptibility to exacerbation. This implies that normal functions for facultative progenitor and stem cells are compromised. Evidence suggests this may be the case from the analysis of biomarkers for epithelial remodelling that are used to follow the severity and progression of lung disease. This study has provided evidence that vitamin A is at least one of the components that promote ATII cells and surfactant secretion, but not Clara cells in postnatal lungs. We know this to be true because there was a decrease of ATII cells synthesis of surfactant in the VAD lung group,

but expression levels of Clara cells seemed in part, unaffected. ATII cells not only serve to maintain lung epithelial cells, but also provide surfactant to reduce surface tension of the air/liquid interface lining the alveoli and enabling respiration. Vitamin A seems to play an important role in regulating surfactant synthesis.

During lung epithelial injury, ATII cells proliferate and then differentiate into ATI cells that replace the injured cells and restore the alveoli. Vitamin A is thought to be necessary for this process. The precise mechanism for the action of vitamin A is not known, but RA appears to be involved. There was no high proliferative activity in SP-C-positive ATII cells in the VAD lung group, signifying that vitamin A initiates ATII proliferation (Tei, Moccia et al. 1999). It is possible that all SP-C expressing ATII cells have the potential of being transformed, but lung injury may only initiate a small population of uncharacterised ATII cells for instance, those expressing low levels of SP-C and presumably processing proliferative potential in various conditions or those residing at specific anatomical locations. Without markers currently available to define subpopulations of ATII cells, it is not possible to assess transformation potential of distinct subpopulations of ATII cells, if they do exist. It is also uncertain how the transformation potential of ATII cells is compared to other lung epithelial cells such as Clara cells, BASCs, ciliated cells, goblet cells and ATI cells under various conditions. Perhaps distinct cell types are susceptible to specific insults and a more in depth investigation is required to delineate the molecular basis on proliferative potential amongst distinct lung cell types or populations.

The role of vitamin A in preventing inflammation is related in part to its interaction with leukocytes. Inflammation occurs in the microcirculation and can be characterised by the movement of fluid and leukocytes from the blood to the extravascular tissue. Vitamin A appears to be most effective against the most prevalent proinflammatory leukocyte, the neutrophil. Vitamin A deficiency appeared to initiate innate immunity because we observed macrophages in the VAD lungs which are known to help initiate specific defence mechanisms. These cells also express the phenotypes, M1 killer and M2 macrophages which are involved in tissue repair. It is more likely that the macrophages seen in our VAD rat lungs were M1 because structurally, they appear oval in shape, whereas M2 are more elongated. The macrophages we observed in the alveolar epithelia were oval. Also M2 are thought to be less inflammatory and less active in the killing of bacteria (Rosas, Thomas et al. 2010, Moreira and Hogaboam 2011). However, investigations of these macrophages were not examined, but this would be an interesting aspect to peruse.

Levels of CC10-positive Clara cells in the bronchiole airway epithelia are reduced in chronic lung diseases such as asthma and CF. However this is not true in our VAD rat model where we only saw a reduction when compared with control groups, therefore we concluded that there was not any differences in the expression of these cells in the bronchioles, even though statistical analysis signified a slight significant difference. What we demonstrated in an environment depleted of vitamin A, CC10-positive cells are still present throughout the tracheobronchial tree of postnatal rats suggesting that epithelial remodelling may involve altered differentiation or subset of (variant) of Clara cells critical bronchiolar epithelial recovery because these CC10-positice Clara cell structure was dissimilar to CC10-positive cells in control

lungs. This supports the notion that there exists a population of bronchial epithelial cells that are CC10-positive in rats that maybe a subset of Clara and non-Clara cells (Bolton, Pinnion et al. 2008). Clara cells in control groups displayed a classical dome-shape, apical protrusion (cap), whereas VAD groups appeared bigger with no dome shaped top. Also, we found that PCNA-positive cells in the bronchiole epithelia to be restricted mainly to the basal cells, indicating that bronchial CC10-positive Clara cells do not serve a proliferative stem cell-like role like true Clara cells. Many studies have shown the expression of Clara cells in the alveolar epithelia as well as in the bronchioles, but in our study, we did not observe this. This, however does not suggest that these cells demonstrated are not "true" Clara cells, but suggests that they may have slightly different functions, according to their location and structure and also bearing in mind that the epithelia has been dramatically damaged. It is interesting to speculate that it may be owing to the Clara cell's defensive role of the epithelia the high numbers present in the VAD lungs.

Nkx2.1is a critical determinant of the lung's epithelium gene expression and lung differentiation (Lazzaro, Price et al. 1991, Bohinski, Di Lauro et al. 1994, Bruno, Bohinski et al. 1995, Stahlman, Gray et al. 1996). Through immunohistochemistry and immunoblots, it was shown to have a critical role in SP-C expression, but not for the Clara cells. The conclusion of this was driven by the fact that when SP-C expression was downregulated in the VAD lungs, TTF-1/Nkx.2.1 expression was also downregulated, whereas CC10 expression did not alter. This supports the role of NKx2.1 in protein expression in relation to respiratory alveolar epithelium in that it is one of the driven forces of ATII activation. Clara cell expression maybe independent of TTF-1/Nkx.2.1 regulation, postnatally, or that the Clara cells present

are in fact variants and may be regulated through another pathway. Through the pattern of staining, it would appear that TTF-1/Nkx2.1 was not detected in ATI cells or in ciliated cells in the conducting airways, suggesting that the differentiation of the subsets of cells from progenitor cells is associated with the loss of TTF-1/Nkx2.1 expression, since ATI cells are generated by terminal differentiation from ATII cells. TTF-1/Nkx.2.1 staining was detected primarily in the nuclei as was SP-C positive ATII cells in control alveoli and was weakly detected in the bronchiole epithelium. However, TTF-1/Nxk.2.1 immunostaining was almost absent in the regions of ATII positivity after injury associated with VAD. TTF-1/Nkx.2.1 immunostaining was not particularly prominent in the bronchiole epithelium of control lungs, although there was staining lining cuboidal cells where regeneration would take place. There was very minimal staining in VAD lungs signifying the importance of vitamin A for normal lung maintenance.

3.2.5: SUMMARY. Understanding the role of airway progenitor cells in lung injury may play in the development or exacerbation of lung disease is critical for the development of cell and molecular therapy. Significant new insights into cellular and molecular mechanisms of epithelial maintenance have been made in recent years that provide insights into the pathophysiology of lung disease. Collectively, our data supports the hypothesis that Nkx2.1 and ATII cells and vitamin A are crucial elements in the homeostasis of the lung, postnatally.

CHAPTER 3.3

EXPRESSION OF MUCINS IN VAD POSTNATAL RAT LUNGS

OBJECTIVE: Vitamin A deficiency (VAD) alters the phenotype of airway epithelium and attenuates the epithelial defense system. Many studies have reported an association of VAD with respiratory disease. In this study, we investigated changes of glycoprotein secretion in a VAD postnatal rat model. Areas of the bronchiole epithelium where goblet cells are present were evaluated in both VAD and control animals. Upon histological evaluation, we found there was a significant increase in the level of glycoprotein secretion in the VAD rat compared with the control rat. When examining secretory levels of the major mucin proteins and those associated with respiratory diseases, we found that there was a significant increase in MUC2 and MUC5B and a relative increase in MUC7 protein in VAD animals. There did not appear to be a substantial change in MUC5AC secretion between the two groups. In this study we concluded that we found mucus hypersecretion along with hyperplasia and metaplasia in VAD rat lungs and that the increase in MUC2, MUC5B and MUC7 may contribute to the susceptibility to airway infection linked to VAD suggesting dysfunction in lung function.

3.3.1: INTRODUCTION: The day to day bombardment of foreign particles inhaled from the environment into the airways can be a hindrance for the normal functioning of the respiratory system by damaging the lining of the epithelium along the lungs. One way in which the lung is able to combat such an onslaught is through the secretion and mechanism of a viscous substance known as mucus, produced by goblet cells embedded in the epithelium of the respiratory tree (bronchi and bronchioles, Fig 3.1).

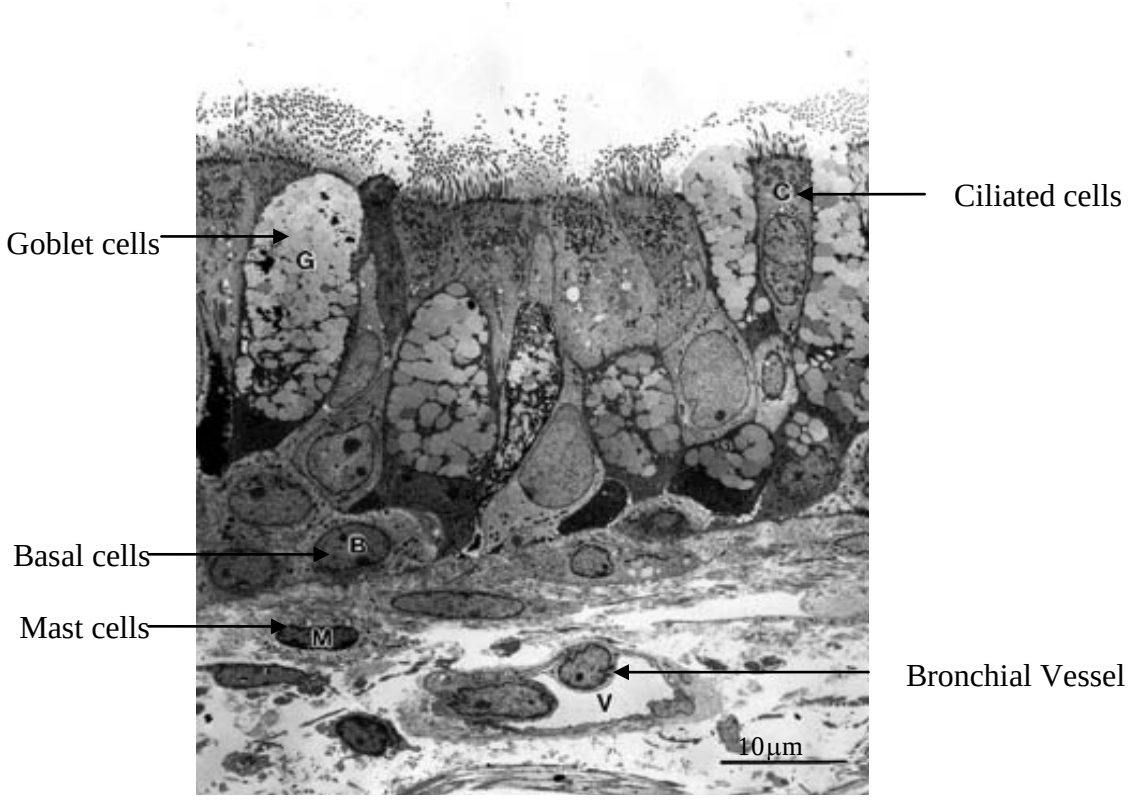

Fig 3.3.1: Transmission electron micrograph (TEM) showing through the surface Mucus enriched in glycoproteins and water which gives it its viscous characteristics. *Rogers. 2008*

Mucus removes unwanted foreign airway particles that enter the respiratory tree by a process known as mucociliary clearance (Vestbo, Prescott et al. 1996, Jeffery and Li 1997, Houtmeyers, Gosselink et al. 1999, Maestrelli, Saetta et al. 2001, Daviskas, Robinson et al. 2002, Rogers 2007). Mucus itself is a non-Newtonian fluid that has both viscous (liquid) and elastic (solid) properties (Rogers DF. 2008, King M. 2006). Viscosity represents energy loss, whereas elasticity represents energy storage. Consequently, the effectiveness of mucociliary clearance has a direct relationship with elasticity, whereby kinetic energy from beating cilia is transmitted to mucus

and an indirect relationship with viscosity (limited loss of energy) (Rogers DF. 2008). Conversely, the effectiveness of coughing has an indirect relationship with elasticity (to limit elastic recoil) and a direct relationship with viscosity and therefore creates an adverse effect on the mechanics of breathing (Rogers DF. 2008, King M. 1989).

Within this non-Newtonian media lay antiseptic enzymes such as lysosomes, immunoglobulins, inorganic salts and proteins such as lactoferrin and glycoproteins (mucopolysaccharides and mucins). These components are secreted by the goblet cells, serving a role in the airway defence.

The histochemical analysis of secretory glycoproteins present in a cell population of normal airway mucosa provides information in regards to changes occurring in a disease state. In human airway disease, secretory hyperplasia is always accompanied by a shift in pH of the heterogeneous population observed in normal circumstances. This shift greatly favours an increase (hypersecretion) in the secretion of acidic glycoproteins which is associated with keratinisation of the mucosa. A change in pH is often observed in airway conditions such as asthma, cystic fibrosis (CF) and COPD, where the function of mucus is greatly changed from a protective role to one that contributes to the respiratory disease (Rogers 2007) (Houtmeyers E et al. 1999, Maestrelli P et al. 2001, Robinson M et al. 2002).

The relationships between mucus hypersecretion and pathological mechanisms of VAD and airway obstruction are poorly understood. Although early epidemiological studies of occupational cohorts failed to associate mucus hypersecretion with rapid progression of VAD, some more recent population-based studies reported an association of chronic mucus hypersecretion with accelerated decline in FEV1 and also an involvement in airway infection (Vestbo et al. 1996, Kim et al 2012). The question of whether mucus hypersecretion cause annoying, but innocuous symptoms or are instead aetiologically related to long-term VAD remains unanswered.

3.3.2: AIM: Chronic mucus hypersecretion along the respiratory mucosa is one consequence of respiratory diseases. Research has shown that depletion of vitamin A is a contributor to such conditions, therefore we would expect to see an increase in the expression of glycoproteins in our VAD animals. Lung sections and homogenized lung tissue from VAD and control animals were used to examine the level of glycoprotein expression secreted from goblet cells. This was achieved by histology (ABPAS staining), immunohistochemistry (MUC2, MUC5AC, MUC5B and MUC7 DAB staining), SDS-PAGE electrophoresis (MUC5B and MUC7 Coomassie/PAS staining) and western blotting (MUC2, MUC5AC, MUC5B and MUC7 immunostaining). SDS PAGE and western blots bands were scanned with ImageJ (downloaded from the National Institute of Health, USA). Band intensity of MUC proteins were compared between VAD and control lungs. These findings were calculated using Student's t-test.

3.3.3: RESULTS.

ABPAS DEMONSTRATION OF GLYCOPROTEIN EXPRESSION: A number of outcomes were concluded from ABPAS staining. Firstly, there was a clear change in pH composition of secretory glycoproteins in VAD animal groups. Expression of these proteins from this group contained a large population of strongly acidic, but very few neutral glycoproteins. Its control counterpart contained a small even heterogeneous population of acidic and neutral glycoproteins (Fig 3.3.2). As expected, its control counterpart contained a small heterogeneous population of acidic and neutral glycoproteins. The total number of positive goblet ABPAS cells was counted to determine hyperplasia in the bronchi epithelial tree. Five non-overlapping fields of bronchioles containing secretory goblet cells were analysed under brightfield microscope. The average cell counts of active secretory goblet cells was calculated using the unpaired student's t-test. Statistical analysis showed that there was an increase in the number of goblet cells secreting glycoproteins in VAD lungs (±) in comparison with control lung groups (±) (Fig 3.3.2). Acidic and neutral glycoproteins were also calculated using the student's t-test. Statistical analysis showed an increase in the number of acidic glycoprotein secretion in VAD lungs (±) and showed a reduction in the number of neutral glycoprotein secretion (±) when compared with secretions levels in control lungs (±) for acidic and (±) for neutral glycoproteins (Fig 3.3.3).

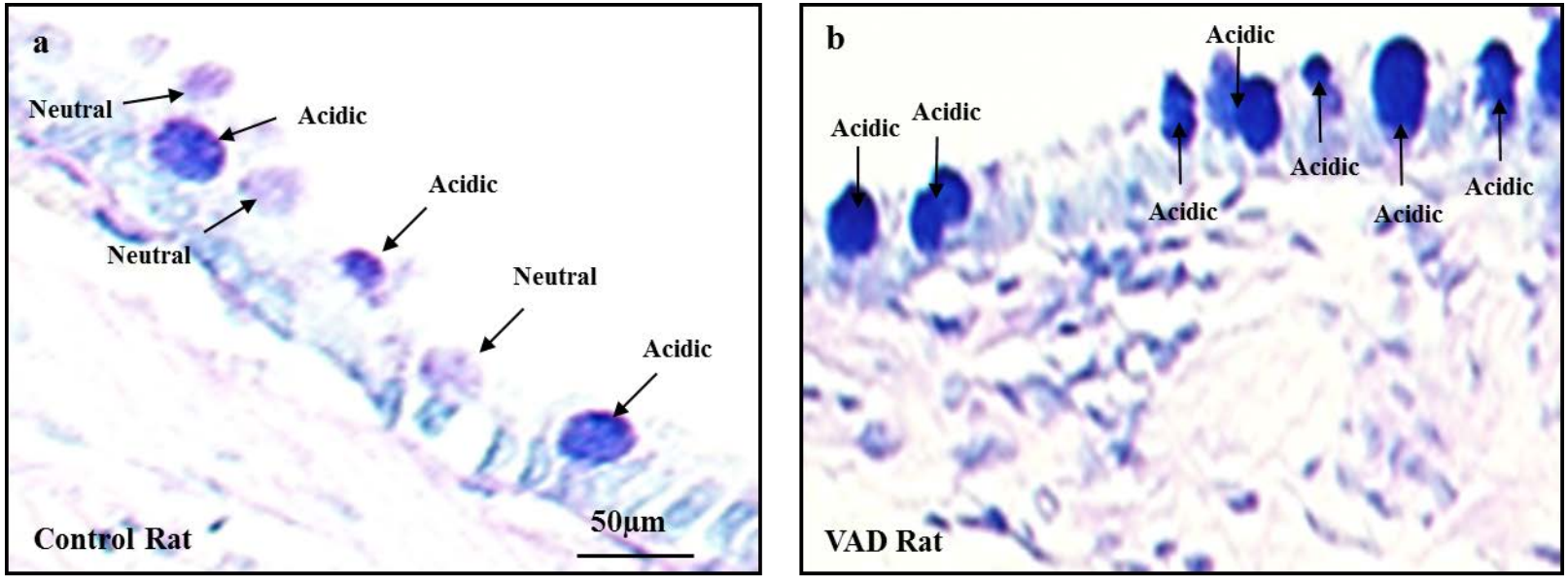

Fig 3.3.2: ABPAS staining demonstrating the localization and secretory distribution of acidic and neutral glycoproteins in control (a) and VAD (b) rat lungs (black arrows). Scale bar: 50µm

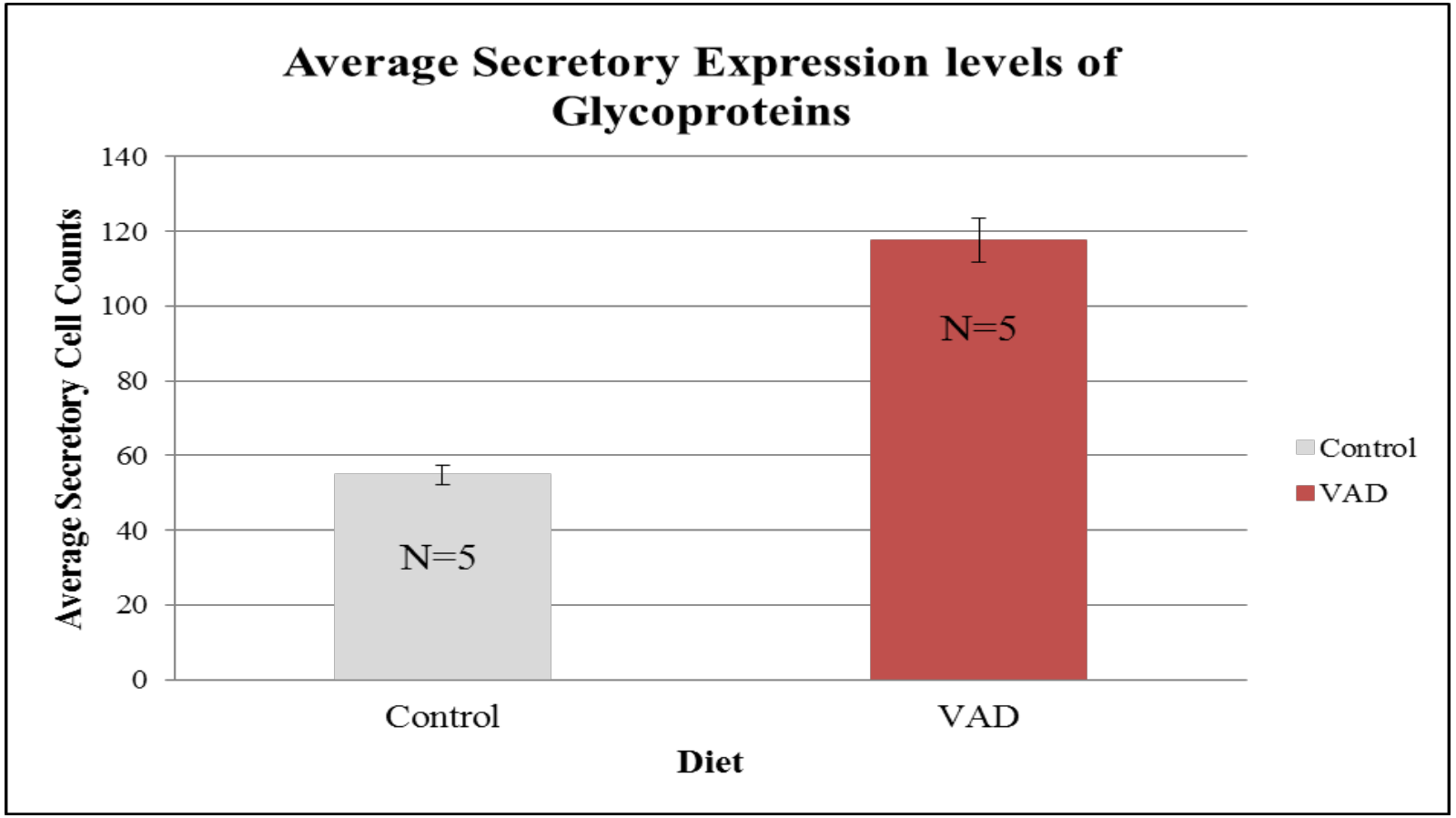

Fig 3.3.3: Histogram of the average number of secretory goblet cells in control (grey) and VAD (red) rat lungs. VAD rat lungs show a higher number of goblet cells secreting glycoproteins in the bronchiole epithelia

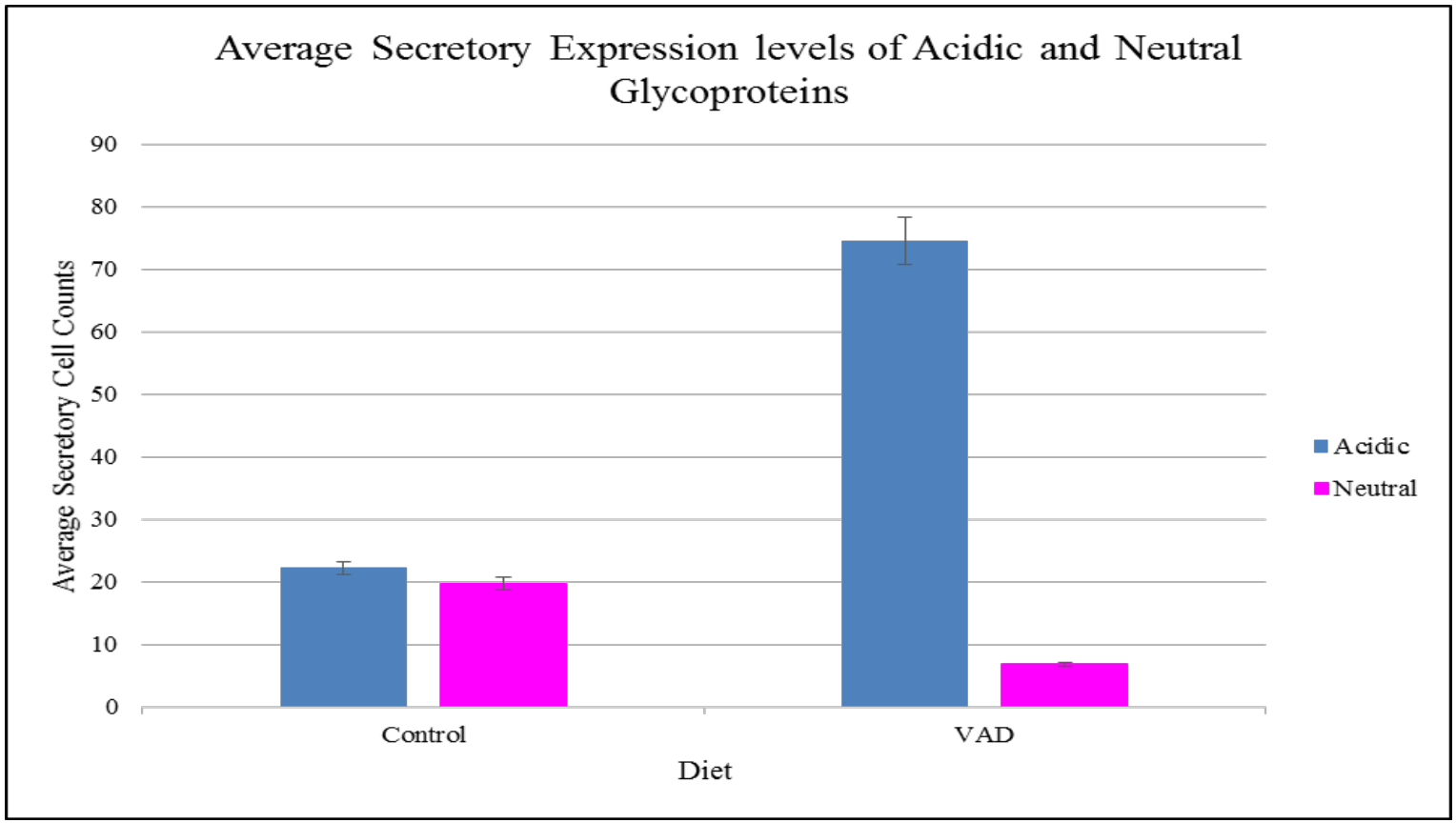

Fig 3.3.4: Acidic and Neutral expression level comparison in control and VAD lungs. Graph displays the amount of acidic glycoproteins has increased substantially in comparison to control lungs giving a strong indication that acidic glycoprotein secretion is strongly stimulated by VAD.

Secondly, the morphology of goblet cells in VAD lungs were examined. They showed the shape of goblet cells in these lung to be columnar rather than oval as observed in control lungs (Fig 3.3.5a and b).

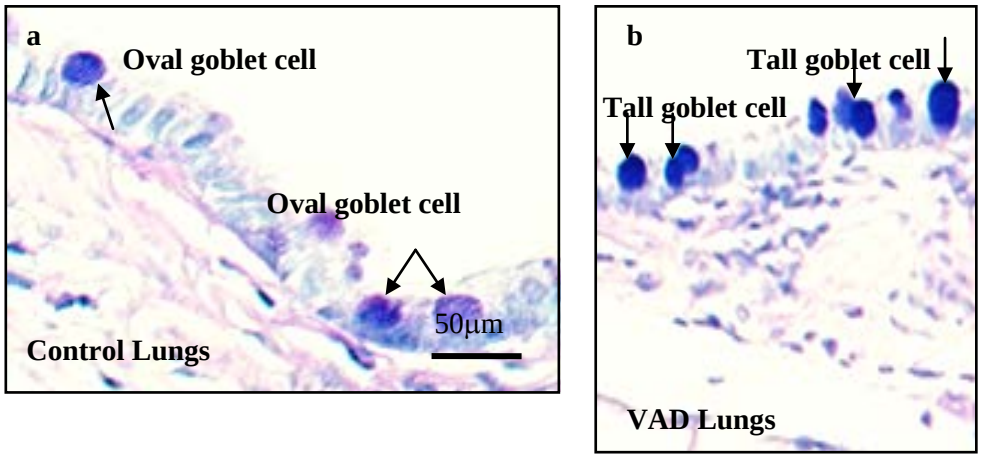

Fig 3.3.5: ABPAS demonstration of secreted glycoproteins in goblet cells. Black arrows in figure (a) and (b) point to the changes in goblet cell structure in control and VAD lungs.

Thirdly, VAD lungs showed frequently populated secretions of acidic glycoproteins along the respiratory tree compared with control lungs, where secretions were sparse confirming that VAD diet exacerbate damage to the bronchiole epithelium by causing a change in the level of acidic glycoprotein secretion most likely caused by inflammation and bacterial infection (Fig 3.3.6).

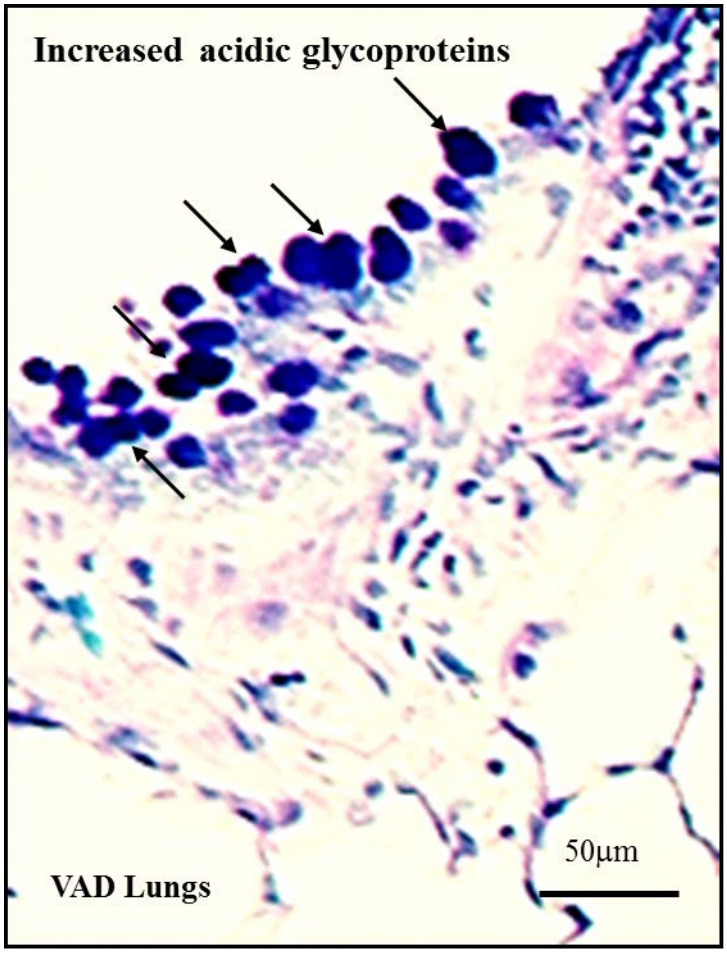

Fig 3.3.6: Increased number of acidic glycoprotein secretion in postnatal VAD rat lung. Bronchiole epithelium also appears damaged suggesting inflammation caused be dietary VAD. ABPAS histochemical staining. Scale bar: 50µm

Next we evaluated the expression levels of the two major mucin proteins (MUC) in the respiratory system; MUC5AC and MUC5B along with two associated MUC proteins with airway disease, MUC2 and MUC7. The measurement of secretory mucins, MUC5B and MUC7 from lung homogenates was examined by comparing Coomassie-PAS stained SDS gels of VAD (Lane 2) and control (Lane 1) samples

(Fig 3.3.7). MUC5B and MUC7 proteins were measured by calculating the band intensity using ImageJ analysis programme. The major mucin protein found in VAD lungs was MUC5B (Lane 2, top band). As expected, a low levels of MUC5B was expressed in control lungs (Lane 1, top band). MUC7 staining was harder to detect, but there was a slight increase in the protein in VAD lung homogenates (Lane 2, bottom band). There was a faint expression of MUC7 in control samples (Lane 1, bottom lane).

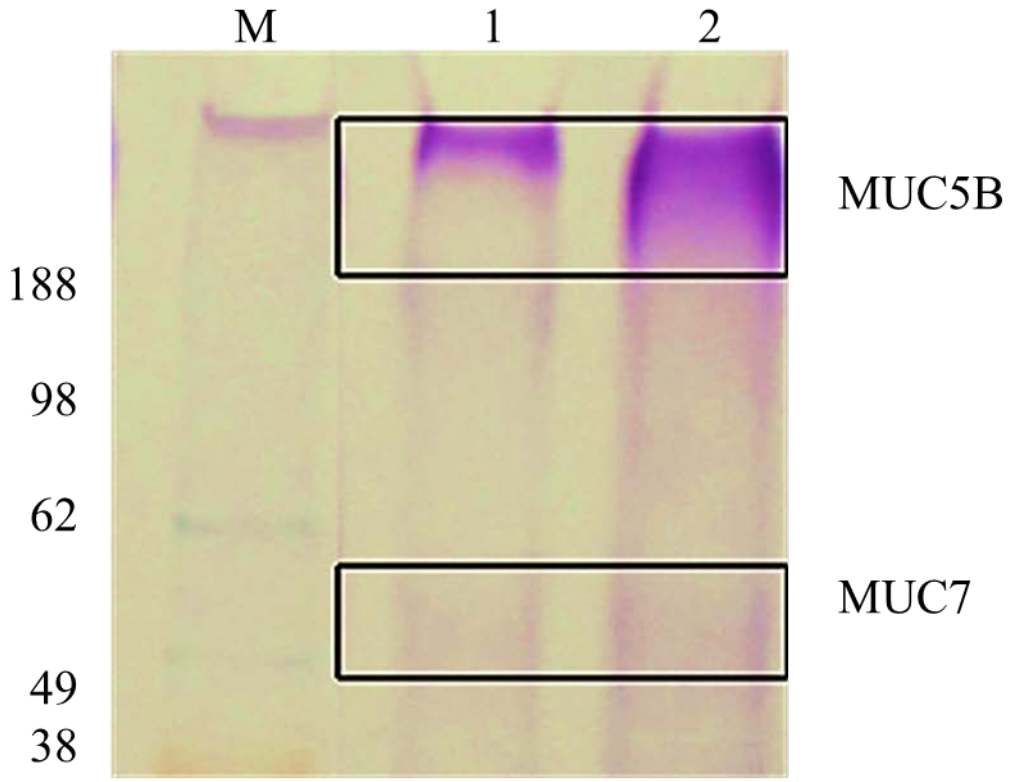

Fig 3.3.7: SDS-PAGE gel electrophoresis on homogenised lungs samples from control (lane 1) and VAD (lane 2) animals. Gels were stained with Coomassie Brilliant Blue R250 (CBB) and then periodic acid Schiff's reagent (PAS). Lanes 1 and 2 were loaded with 15µl of sample. M = Marker

Calculations confirmed visualised observations of stained MUC bands. MUC5B glycoprotein showed that there was a significant increase in VAD homogenate lung samples (one-way AVONA), but no significant increase with MUC7 in VAD homogenates compared with control samples (Fig 3.3.8, Table 3.3.1). This indicated that MUC5B and not MUC7 contributes to mucus hypersecretion initiated by dietary VAD.

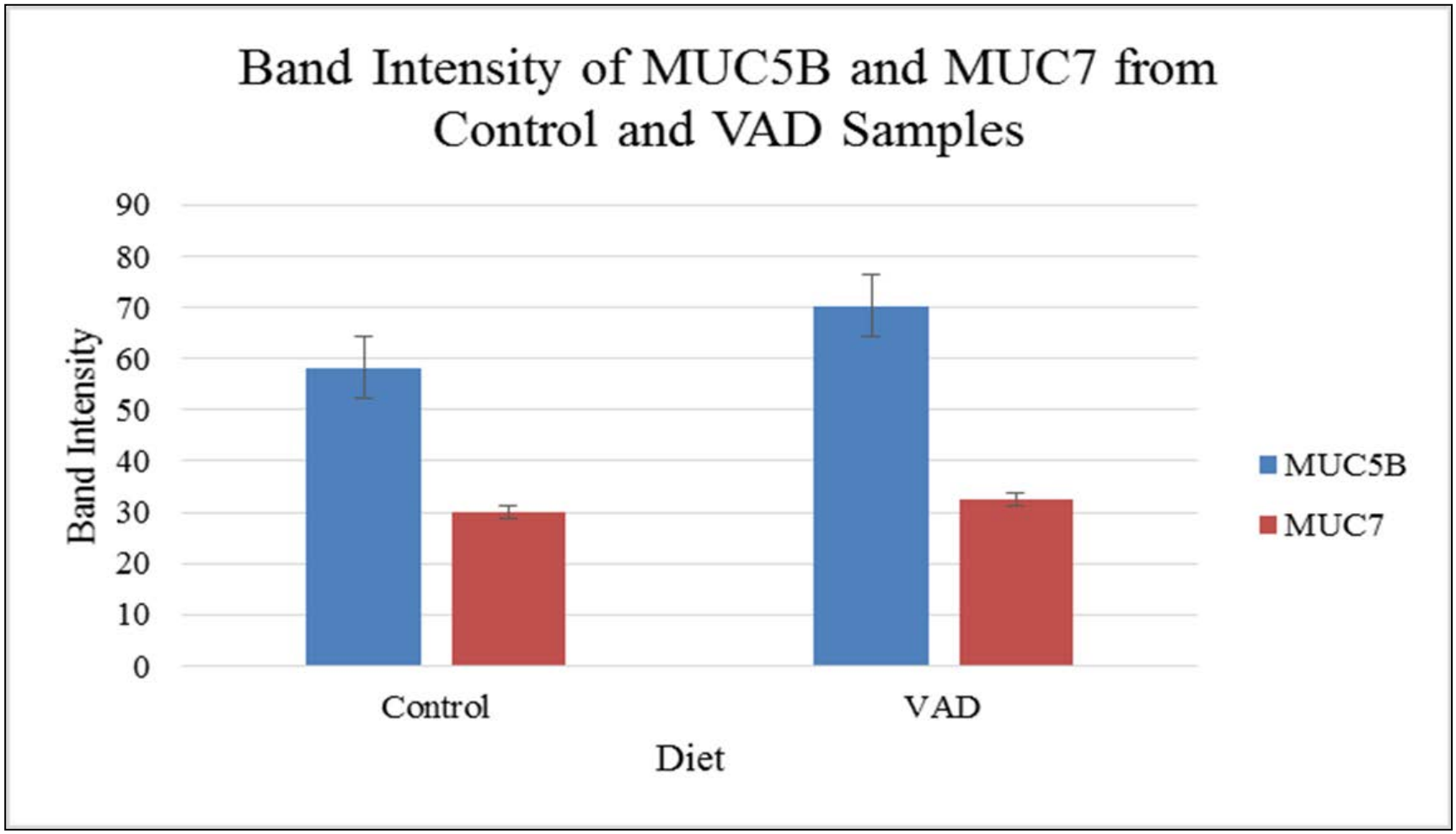

Fig 3.3.8: Band intensity of MUC5B and MUC7 proteins in control and VAD lung homogenates. Graph displays an increase of MUC5B secretion in VAD lungs, but an unchanged level of MUC7 secretion when compared with control samples.

Table 3.3.1: MUC protein expression in VAD and control homogenates. Results are expressed as a percentage of MUC levels in control and VAD ± standard error of the mean. One-way AVONA indicated whether there was a difference in diet groups. * Indicates the proteins significant difference from VAD samples after t-test, where $P<0.05$ followed by the Bonferroni correction where $P<0.016$.

MUC Proteins	n	Control	VAD	ANOVA Results
MUC5B	5	58.2 ± 15.0	70.3* ± 4.6	P<0.05
MUC7	5	30.0 ± 6.3	32.5 ± 4.1	N.S

Immunohistochemistry of MUC Expression: All four MUC proteins were stained on 5µm sections taken from control and VAD lungs and analysed using a brightfield microscope. MUC5AC and MUC5B expression was only detected in the bronchiole airway epithelium, whereas both MUC2 and MUC7 expression was detected in both alveolar and bronchiole airway epithelium (Fig 3.3.9a - l). Sections from VAD lungs showed an increase of MUC2, MUC7 (alveolar and bronchiole epithelium) and MUC5B and MUC7 (bronchiole epithelium), figs b, d, h, j and l. There did not appear to any change in the level of expression with MUC5AC (bronchiole epithelium), figs f. Expression pattern of MUC2 staining was distinctly different in VAD airways compared with control airways (a - d). In VAD alveolar epithelium (b), MUC2 staining showed nuclear overexpression of the protein, whereas MUC2 expression in control alveolar epithelium showed clear nuclear staining. Protein expression showed a clear outline of each positively stained nuclei (a) and was less in number compared with MUC2 expression in VAD alveolar epithelium. MUC2 protein in the bronchiole epithelium of VAD lungs (d) also showed overexpression compared with bronchiole epithelium in control lungs (c). Expression of this protein was mostly prominent on top of goblet cells forming a “cap”. Expression of MUC2 positivity showed granulation in these caps. There was also expression located long the basement goblet cells on the epithelium. In control bronchioles, positive MUC2 staining appeared flat on the surface of goblet cells with very little granulation. Expression of MUC2 was also seen on the basement of goblet cells, but unlike expression seen in VAD bronchioles, expression was intermittent in control bronchioles. The average cell counts of MUC2 and MUC7 in alveolar airways of VAD and control lungs were as follows; 84.5 ± 3cm³ (VAD) v 52.3 ± 2cm³ (control) for MUC2 and 26.3 ± 4cm³ (VAD) v 36.6 ± 9cm³ (control). Average cell counts for

MUC2 in bronchiole airways were 67.9 ± 4cm³ (VAD) v 29.2 ± 6cm³ (control); MUC7 average counts were 27.8 ± 1cm³ (VAD) v 63.5 ± 5cm³ (control). Next we examined MUC5AC and MUC5B protein expression. These proteins were only detectable in the bronchiole airway epithelium (e-h). Both pattern of MUC5AC staining was intense, but clearly defined goblet cell structure (e and f). Goblet cells in VAD lungs did appear granular, whereas some, but not all goblet cells in control lungs gave a granular appearance. Staining pattern of MUC5AC surrounded the whole of goblet cells. The caps situated on top of the goblet cells in VAD lungs appeared to show more MUC5AC expression and was more intense. Average cell counts for MUC5AC was 111.0 ± 2cm³ (VAD) v 102.3 ± 8cm³. MUC5B expression in VAD bronchiole epithelium (g) showed an increase compared to MUC5B expression in control bronchiole epithelium (h). Unlike the pattern of staining seen in MUC5AC where goblet cells were clearly defined in both control and VAD lungs, staining of MUC5B in VAD bronchioles showed ill-defined goblet cells. MUC5B staining showed its characteristic cap feature in goblet cells in control bronchioles. The average cell counts of MUC5B expression was 100.8 ± 7cm³ (VAD) v 68.1 ± 4cm³ (control). A summary of MUC protein expression in both alveolar and bronchiole airway epithelium are shown in fig 3.3.10 and fig 3.3.11 and calculations listed in table 3.3.2.

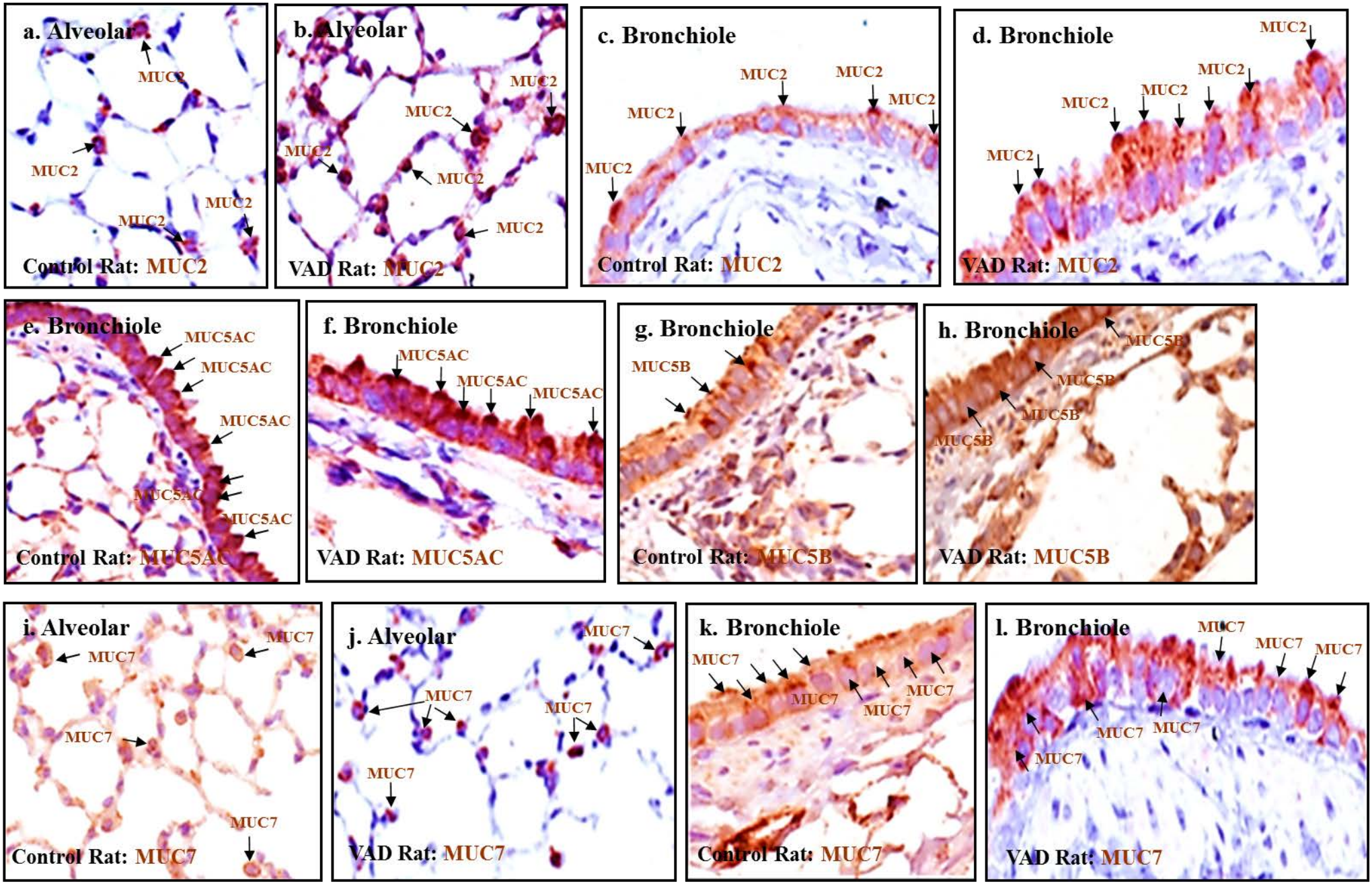

Fig 3.3.9: Immunohistochemical staining demonstrating MUC2, 5AC, 5B and 7 protein in alveolar and bronchiole airway epithelia

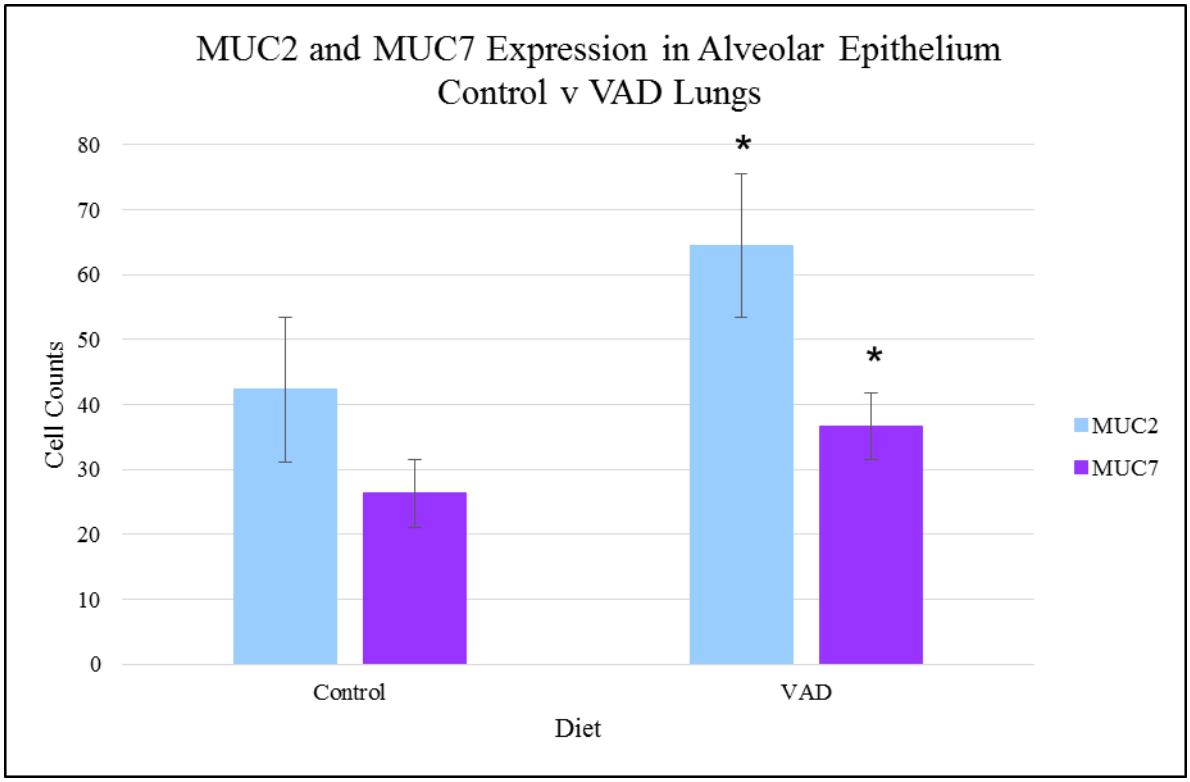

Fig 3.3.10: Graph shows the average number of MUC2 and MUC7 protein expression in the alveolar airway epithelium of VAD lung groups compared with control lung groups. Here the graph shows an increase in MUC2 and a slight increase in MUC7 in VAD lungs indicating that increase of MUC2 and MUC7 secretion is promoted by VAD diet.

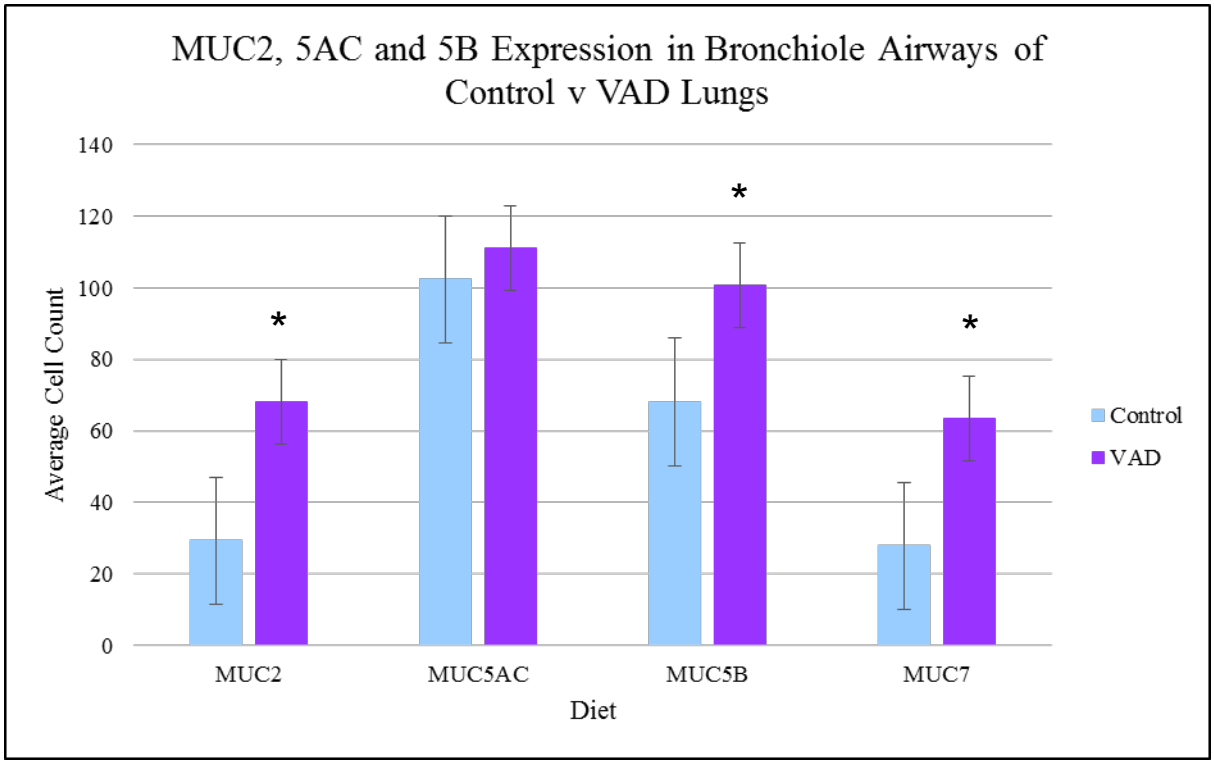

Fig 3.3.11: Graph showing the average number of MUC2, MUC5AC, MUC5B and MUC7 expression in goblet cells in the bronchiole airway epithelium of control and VAD lungs. There is a clear indication that the expression levels of MUC2, MUC5B and MUC7 have an increased response to VAD diet and that MUC5AC expression levels is mostly unaffected.

Western blots shown in figure 3.3.12 are representative of lung homogenate samples extracted from control and VAD animals. Lung homogenates from these groups of animals were stained with MUC proteins previously used for IHC detection (Fig 3.3.9). All four MUC proteins were present in VAD and control homogenate samples. In VAD samples (Lane 2), MUC2, MUC5B and MUC7 proteins were strongly detected and showed upregulation compared with these proteins detected in control samples (Lane 1), however there appeared to be no change in MUC5B

expression levels between VAD and control samples. The WB results appeared to collaborate with staining results obtained from IHC staining (Fig 3.3.9).

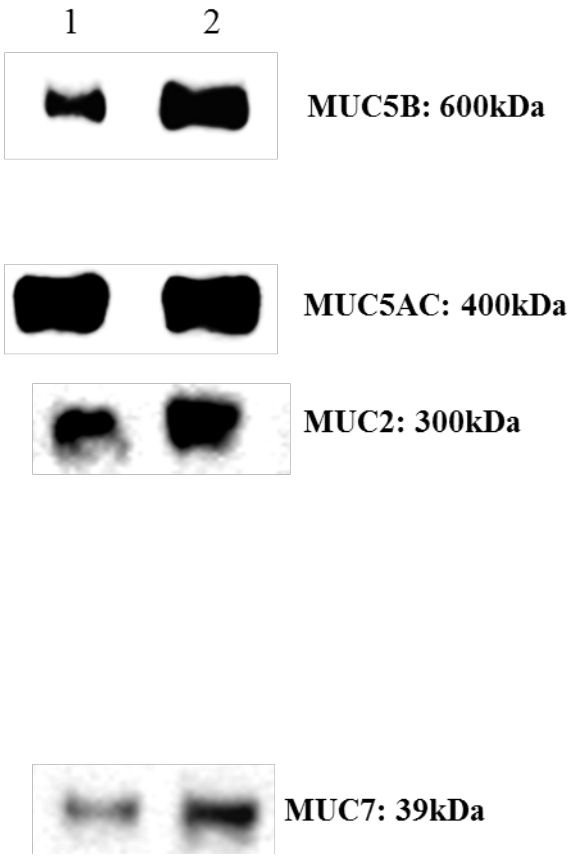

Fig 3.3.12: Western blot analysis of MUC proteins in lung homogenates of control (Lane 1) and VAD (Lane 2) animals. WB were probed with antibodies against MUC2, MUC5AC, MUC5B and MUC7 proteins. Molecular weights varied from 39-600kDa.

3.3.4: DISCUSSION. The study in this chapter was performed to gain knowledge about the way in which deficiency of vitamin A contributes to mucus hypersecretion of the airways. We used a VAD postnatal rat model to highlight the importance of vitamin A for mucus homeostasis.

Vitamin A depletion affects goblet cells in various ways depending on the tissue or species. Hypersecretion is accompanied by goblet cell hyperplasia and glycoprotein metaplasia. Chen et al. found that an increase in goblet cell number is due to changes of stored and secreted mucus. In our study, after staining tissue sections with ABPAS we found that VAD lungs showed goblet cell hyperplasia and metaplasia (Fig 3.3.2). In VAD lungs there was a striking change in what would be described as "normal" heterogeneity of glycoproteins in the bronchiole airway epithelium in healthy bronchiole airways. The use of ABPAS staining allowed us to observe a transformation of a low heterogeneity population of glycoprotein secretion to a population that consisted mainly of large population of acidic glycoproteins. Previous experiments using irritated lungs by sulphur dioxide and tobacco smoke also demonstrated the same changes seen in VAD lungs (Jeffery et al. 1997). There is literature concerning studies *in vivo* that strongly suggests vitamin A is involved in the process of glycosylation (Chan et al. 1987, Rosso et al. 1981). pH change of glycoproteins seen in VAD lungs could be caused by an alteration in the process of glycosylation with the removal of specific glycan linkages, such as highly reproducible reduction in some sialic acid linkages following immune activation of mammalian lymphocytes.

Secreted high molecular weight mucin is important in many functions performed in the bronchiole airways and in this study this was clearly demonstrated by IHC staining and immunoblotting lung samples of VAD and control animals. We focused on four airway mucins associated with airway diseases, MUC2, MUC5AC, MUC5B and MUC7. MUC5B and MUC7 were semiquantified by scanning PAS-stained gels because this method proved to be reproducible. There was variation between the two animal groups in their relative amounts of secreted mucins produced and this reflected the significant difference amongst MUC2, MUC5B and MUC7 in VAD lung samples. We observed an increase in MUC2 expression in the alveolar and bronchiole airway epithelia of VAD lungs (Fig 3.3.9B and D). These results were similar to a study where MUC2 mRNA was shown to be upregulated during inflammation or bacterial infection initiated by vitamin A deficiency (Guzman et al. 1996). This study alongside our postnatal rat model suggests that MUC2 may play an important role in airway mucus hypersecretion. However, we did not see a change in the expression levels with MUC5AC protein between VAD and control samples. There may be a few explanations as to why this is so; first MUC5AC may not be involved in mucus hypersecretion of the VAD pathway and second; MUC5AC may respond differently in diseased rat lungs from other species such as human lungs. In the human lung, MUC5B is expressed in glands, but in rat lungs used in this study, MUC5B was located in goblet cells (Fig 3.3.9g and h), (Davies et al. 1999, Reid et al. 1997). Like MUC5AC, patients with chronic airway conditions show an upregulation of MUC5 (Burgel et al. 2007, Caramori et al. 2004, Groneberg et al. 2002a, Kamino et al. 2005, Kirkham et al. 2002). In sputum obtained from COPD airways, the abundance of MUC5B exceeds that of MUC5AC, a pattern which is reversed in healthy airways (Kirkham et al. 2008). An increase of MUC5B

secretion demonstrated by IHC was also observed in our VAD lungs (Fig 3.3.9g and h) and was confirmed by protein levels detected by immunoblots and statistical analysis (Fig 3.3.11 and 3.3.12). It could be argued that an increase from this protein was in response to inflammation and perhaps an increase in bacterial activity. These levels could also have been a counteractive response to increasing secretion of MUC2 which is also associated with increase in bacterial infections in the respiratory and gastrointestinal tracts. Finally, we examined expression levels of MUC7 in VAD lungs. MUC7 has been reported to be expressed in the trachea and salivary glands containing acinar cells by Northern blot and RT-PCR analysis (Iwase et al. 2002). In our study, MUC7 was found to be expressed in low levels in both alveolar and bronchiole airway epithelium in control rat lungs. It was shown to be elevated, to some degree in VAD rat lungs of both located areas (Fig 3.3.9 i-l). Cell counts gave a significant value of $P>0.05$ for both epitheliums, signifying MUC7 response to depletion of vitamin A. This data was also confirmed by immunoblot analysis (Fig 3.3.12), where expression levels of this protein was upregulated. These abnormal levels of MUC7 expression could due to its functional role as protective, much like MUC2 and MUC5B. Upregulation of these mucins suggest modulation of structure of protein in VAD lungs.

The contribution of mucus hypersecretion to airflow limitation is problematic as an array of mechanisms leading to this process is still being discovered (Rogers 2007, Kirkham et al. 2002). Different MUC proteins appear to be present in the respiratory system of VAD lungs. MUC5AC and MUC5B are the major MUC proteins in airway secretions, but generally there is more MUC5B in respiratory disease than in MUC5AC.

The importance of MUC5B glycoproteins in inflammatory airway diseases such as VAD and asthma is unclear, but it may relate to differences in propensity for bacterial colonisation of the lungs. Like COPD patients, VAD patients are prone to pulmonary infections (Rogers. 2007, Davis. 2001). As observed in our postnatal VAD rat model, MUC2 and MUC7 protein are expressed in control lungs, but in relatively low levels. The distribution of these proteins in comparison to VAD lungs show similar pattern of expression, but are upregulated. It has been reported that both MUC2 and MUC7 mRNA are upregulated in the airways of smokers, but is found in small amounts in COPD patients (Kirkham et al. 2002, Davis et al. 1999). These findings are suggestive in that there are differences in goblet cell phenotype between airway inflammation. Changes in the phenotype of goblet cells suggest changes in mucus viscosity, leading to a change in mucus composition. This in turn altering interactions of mucus with other components of the airway liquid interface. Although these changes are considered to be involved in the pathophysiology of hypersecretion, the relative contribution of each change in relation to disease development and clinical symptoms needs to be resolved.

Facilitating mucus clearance and changing the viscoelasticity of mucus; a change in viscosity and elasticity will depend upon whether mucociliary clearance or coughing is required. Decreasing viscosity may not markedly change mucus clearance by itself and may require the upregulation of surfactant protein, designed to regulate surface tension and therefore important in elastic recoil.

3.3.5: SUMMARY: Patients with chronic airway conditions which include inflammation as part of its clinical feature show an increase of MUC expression on surface goblet cells are likely to affect the composition and biophysical properties of airway mucus. Our data shows that VAD induces mucus hypersecretion in postnatal rat lungs. Interestingly, secretory levels of MUC5B in VAD lungs were much greater than expected. Increased expression of MUC2, 5B and 7 suggest modulation of these proteins caused by depletion of vitamin A because in control lungs there was much reduced expression. Cessation of mucus hypersecretion will not solve the underlying cause of chronic airway disease in VAD patients. Overall, mucus hypersecretion is a clinical feature that needs further investigation via mechanism (s) and transportation of mucus especially as apart from its protection role, it also has a role in the mechanism of elastic recoil.

CHAPTER 4.1

MORPHOLOGY AND MORPHOMETRIC ANALYSIS OF REGENERATIVE RETINOIDS IN POSTNATAL DEXAMETHASOME MOUSE LUNGS

OBJECTIVE: For the study of retinoid regenerative efficacy in lung injury, we experimentally generated an emphysematous-like model using an anti-inflammatory steroid, dexamethasone in postnatal mouse lungs. First we examined the architecture of dexamethasone-treated lungs (its parenchymal components) and measured its alveoli surface area. Like in the VAD postnatal rat lungs (Chapter 3.1), these lungs showed a vast reduced gas-exchanging surface area and an increase in vascularisation. Most importantly, there was an increase in L_m measurements. The second objective was to examine the efficacy of selective retinoid ligands in the process of regeneration. We achieved this by not only comparing these treated lungs with dexamethasone lungs, but also comparing with tRA because this active form of RA has been shown in numerous studies to reverse the effect of experimentally-induced emphysematous-like features in the adult lung. From our study we found that the most effective and efficient retinoid ligand was RARα. Its morphological features and L_m values were the closest to tRA, followed by RARγ, RARβ then lastly pan-RXR. The data generated allowed us to speculate whether singular or combined forms of retinoid ligands work better in reversing damaged lung structure and function.

4.1.1: INTRODUCTION. Chronic Obstructive Pulmonary Disease (COPD) has become a social toll Worldwide and since 2007, is now considered to be the fourth leading cause of mortality in the UK. Through the increase in incidence rates, it has become more evident that there is a requirement for better therapeutic preventions than what is currently available (lung transplantation or oxygen therapy). The problem with therapies such as transplantation is finding a suitable donor which is time consuming and limited to a small population of patients. It is universally agreed that early detection and intervention, as well as far more specific and effective therapies for this debilitating disease is required. One possible way to treat such disease would be to find a way to induce diseased lung tissue to self-regenerate. Unfortunately, a significant barrier for such regenerative therapies is that damaged adult alveolar tissue seems incapable of self-renewal.

There is increasing interest in the mechanism(s) underlying COPD. In mice, there has been an increase investigating the hallmarks of COPD; namely emphysema. To approach this, most studies examining the mechanisms behind emphysema have employed quantitative analyses on histological sections by the use of the mean chord intercept (Chord) length (MILI) or the mean airspace chord length (L_m) technique (Maden 2006). L_m is a useful and popular parameter or peripheral lung structure as it describes the mean distance of airspace (Knudsen, Weibel et al. 2010). The crux of the use of L_m is that it is not considered as a robust measurement of internal lung structure as it is significantly affected by lung volume changes due to simple inflation conditions or to altered elastic recoil (Knudsen, Weibel et al. 2010). Also

L_m measurement is considered to be a measurement of the entire acinar airspace, with alveoli and alveolar ducts combined rather than just a measurement of alveolar size, since probing lines explores the entire inner complexity of the airspace (Knudsen, Weibel 2010). Nevertheless, this measurement is appealing because it gives a simple and direct indication of a structural size and any changes that may have occurred in the lung, be that in human or experimental pathology relevant to human lung disease (Knudsen, Weibel et al. 2010, Hautamaki et al. 1997, Kang et al. 2008, Mall et al. 2008, Morris et al. 2003).

The discovery that alveolar regeneration can be induced by the vitamin A metabolite, RA represents a significant step in the intervention for a potential therapeutic cure for the disease.

The molecular mechanisms behind the induction of alveolar regeneration through agents such as RA and/or its receptor agonist ligands is an important and exciting topic. Current investigations are underway establishing RA and receptor agonist ligands as therapeutic agents for chronic lung diseases. Specific RAR molecules involved in the promotion of regeneration has only recently been identified and on the basis of developmental studies, certain conclusions have been made. All components of RA signalling pathways are present during alveolar development, the binding proteins, RAR synthesising enzymes and finally the RARs and RXR (Hind, Corcoran et al. 2002, Maden 2006).

4.1.2: AIM. Treatment of dexamethasone in postnatal mouse pups was used to generate emphysematous-like features, both morphologically and morphometrically. These lungs also showed reduced gas-exchanging Sa. The difference between this model and previous COPD/emphysematous models was destructive agents were introduced in adult animals after they had fully matured, whereas is dexamethasone was introduced to newborn pups before their lungs were fully matured. Therefore the dexamethasone model is “distruptive” and a “destructive” model. We used a selection of RAR ligand to assess their regenerative properties in dexamethasone lungs via morphology and morphometric analysis. The results are discussed in 4.1.3.

4.1.3: RESULTS. Dexamethasone-treated lungs displayed enlarged alveoli airspace throughout the lung (Fig 4.1.1). Alveoli cell walls appeared thin and in some cases, showed a presence of lymphocytic infiltrates. ATII cells in these cell walls appeared squamous and irregular in shape. There were areas where these cells formed clusters. Like VAD, an increase in vascularisation was also observed. Increase in vascularisation could have been due to oxidative stress due to inefficient anti-oxidant response in these lungs. This damage suggest a contribution to structural and functional impairment. This may contribute to COPD pathology, inducing apoptosis, tissue damage as well as airway inflammation since airway epithelial cells respond to increased ROS levels by producing cytokines/chemokines that promote inflammatory cells to areas of damaged tissue. On average, this group had increased from 68.1± 1.0μm (control group) to 94.3 ± 2.1μm (dexamethasone group, Fig 4.1.1b and d, Table 4.1.1).

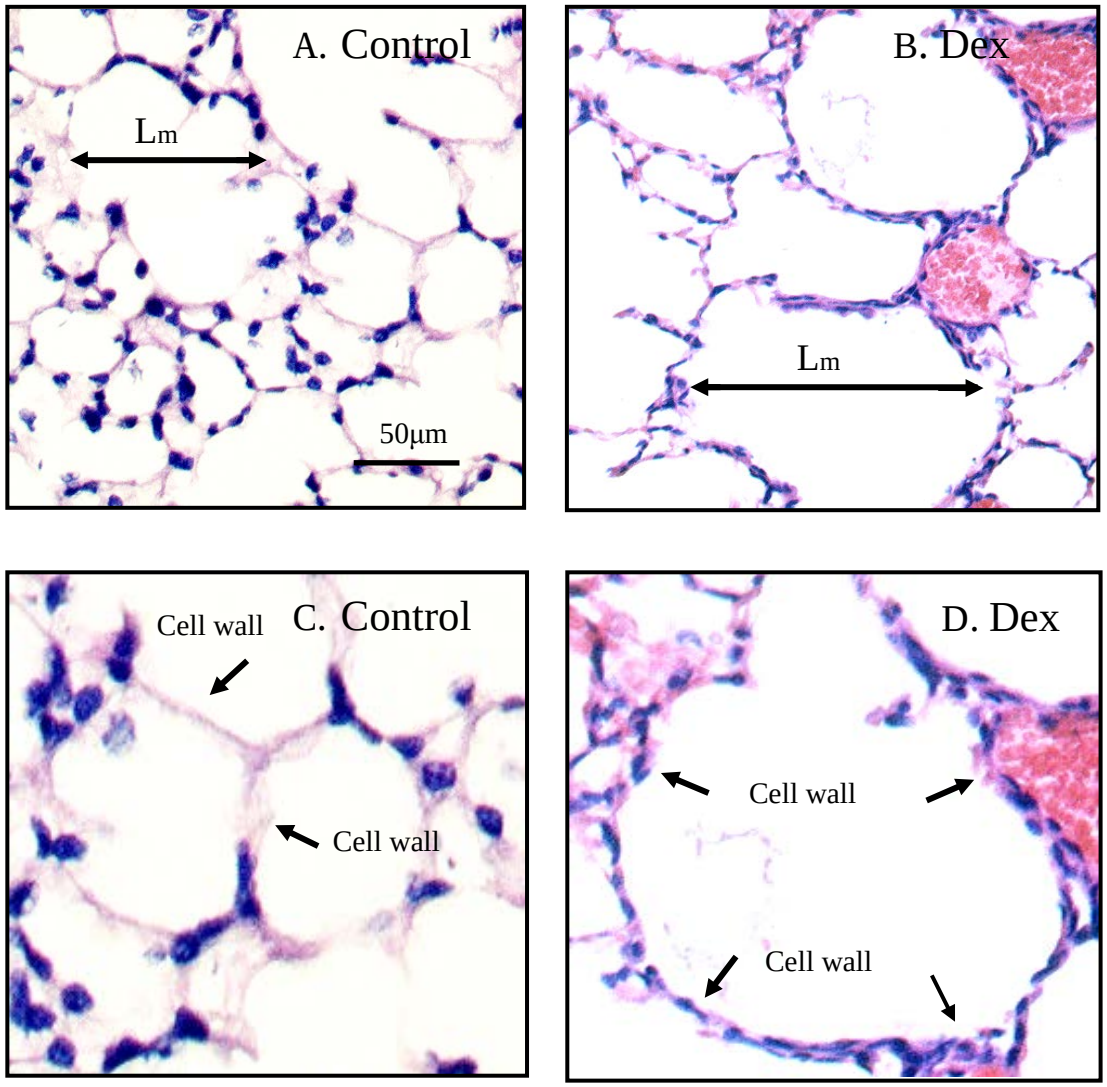

Fig 4.1.1: Morphology and morphometric analysis of dexamethasone lungs (b and d) compared with control lungs (a and c). H&E staining for general morphology. Representative transverse sections show typical structures of alveoli seen in postnatal dexamethasone and control mouse lungs. Alveolar airspace has greatly increased in dexamethasone lungs. Cell walls are much thinner and there is an increase in vascularisation in response to inflammation. Scale bar = 50µm

We examined the effects of tRA as an addition to dexamethasone-treated lungs. tRA was administered to dexamethasone lungs at a concentration of 20mg/ml. These animals were sacrificed at P90 and tRA regenerative efficacies were examined. These lungs were compared with dexamethasone and control lung groups (Fig 4.1.2c). Morphological features of dex-tRA treated lungs showed small pockets of enlarged alveoli, but the majority of these lungs seem to resemble control morphological features (Fig 4.1.2a and c). In comparison with dexamethasone-treated lungs, there appeared to be a marked increase alveolar cell wall thickness and increase in vascularisation. Alveolar septa were short and stumpy in dex-tRA lungs in comparison to dexamethasone lungs whose septae was similar to VAD lungs, long, thin and extended (Chapter 3.1, Fig 4.1.2b and c). This process coincided with an increase of alveolar volume and percentage recovery (Table 4.1.1). The average Lm value for this group was 81.6 ± 2.0μm (Fig 4.17). There was however some variation in regeneration and recovery response in this group. Of the eight animals generated, only two sample lungs showed complete regeneration as defined by L_m values close to control values (69 - 75μm). Two animals showed no response (L_m>85μm) so in terms of percentage regeneration, dex-tRA lungs showing complete regeneration gave a value of 75%, whereas dextRA lungs showing no response gave a value of only 25%. The mean Sa of dex-tRA groups showed a slight increase of 345.4 ± 18.1cm² compared with dexamethasone group whose value was 305.5 ± 11cm². Even though there was a slight difference between dex-tRA and control groups (345.4 ± 18.1 and 434.0 ± 8) respectively (Table 4.1.1).

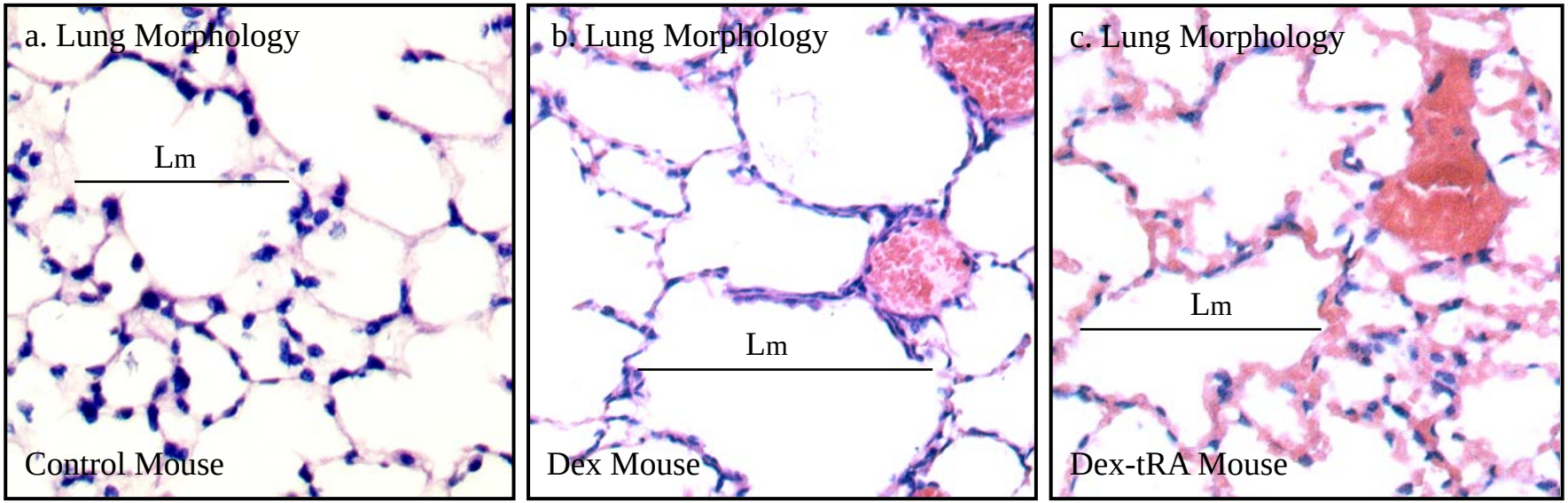

Fig 4.1.2a-c: Lm. Morphology of postnatal mouse lung (H&E staining). Representative transverse sections show typical structure of alveoli seen in postnatal dex-tRA mouse lungs (c) compared with dexamethasone (b) and control mouse lungs (a). Black lines represent where the measurements for Lm are calculated. Scale bar = 50µm

We examined the regenerative capacity of the ligand receptor agonists also administered in dexamethasone lungs. These animals were given a concentration dose of 2mg/ml, 10 times less than dex-tRA animals. This level was deemed to be less toxic for the ligands than the 20mg/ml dose used for tRA. These ligand groups were compared to regenerative properties of tRA. The first ligand to be examined was RARα. Fig 4.1.3a - c shows representative structural features of dex-RARα. H&E staining of these lungs demonstrated similar regenerative morphology and morphometric response to dex-tRA treatment. There was a reduction in vascularisation which would suggest in a positive response to inflammation and in the levels of cytokine/chemokine activity. In terms of L_m values, this group produced similar values as tRA lung groups (82.8 ± 3μm, Table 4.1.1). However, dex-RARα gave a slightly less recovery rate value than dex-tRA; 44%. The overall significant value of dex-RARα compared with dex-tRA was greater than 0.05 confirming similarities in regenerative efficacies.

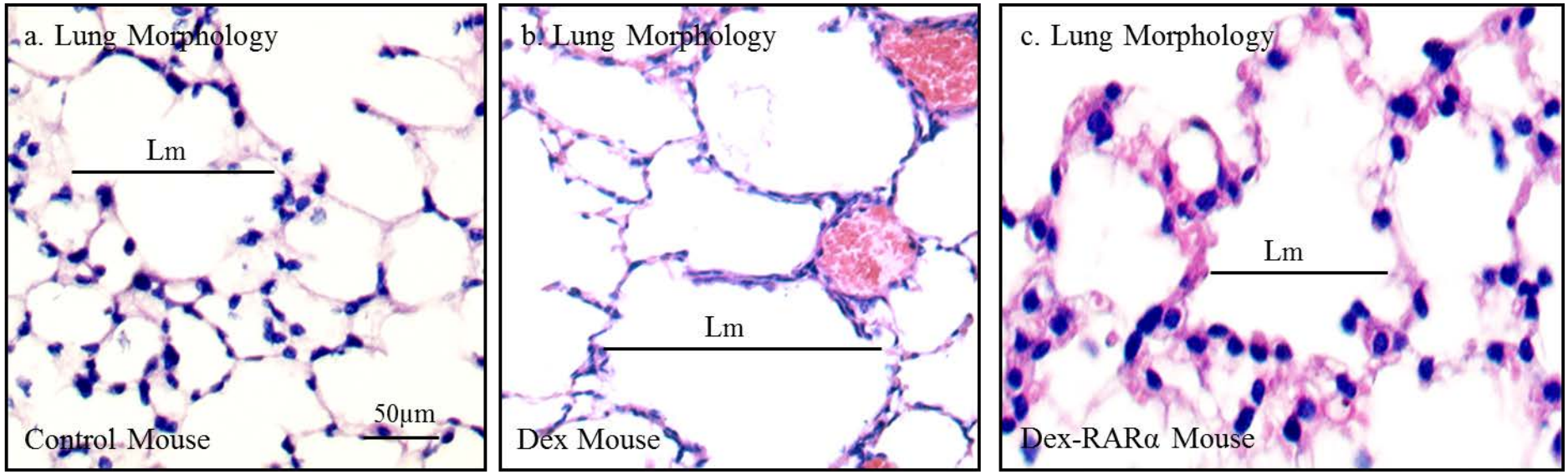

Fig 4.1.3a-c: Lm. Morphology of postnatal mouse lung (H&E staining). Representative transverse sections show typical structure of alveoli seen in postnatal dex-RARα mouse lungs (c) compared with dexamethasone (b) and control mouse lungs (a). Black lines represent where the measurements for Lm are calculated. Cell walls of dexRARα lungs (c) appeared thicker than control lungs (a) Scale bar = 50µm

Next we examined the regenerative efficiency of the induction of RARβ in dexamethasone-induced emphysema. The addition of this receptor was shown to have no morphological or morphometric improvement from dexamethasone-treated lungs (Fig 4.1.4b and c). H&E staining demonstrated a strong morphological similarity between dex-RARβ and dexamethasone lung groups. This receptor group showed enlarged airspace with thin cell walls and there was a slight increase in vascularisation when compared with control lug groups (Fig 4.1.4a - c). Their alveolar septae were short and stumpy. The average L_m values of this group was 84.6 ± 2.3μm (Table 4.1.1). In terms of its recovery, this group gave a percentage value rate less than dex-tRA and dex-RARα lung groups which was 49%. However, dex-RARβ L_m values were closest to dex-RARα whose values were 82.8 ± 3.0μm, 44% recovery (Table 4.1.1). This group also produced lower Sa average value of 274 ± 13.8cm², which in comparison with dex-tRA of 345.4 ± 18.1cm² gave a difference of 70.6cm² and dex- RARα (302.2 ± 18.5) gave a difference of 27.4cm². Comparing dex-RARβ group with control groups (434.7 ±7.5cm²) gave a considerable difference of 159.9cm². This difference was even higher than control versus dex values (305.5 ± 10.5cm²) which gave a difference of 31.3cm². So in summary of this retinoid performance, dex-RARβ gave poor performance in terms of regeneration.

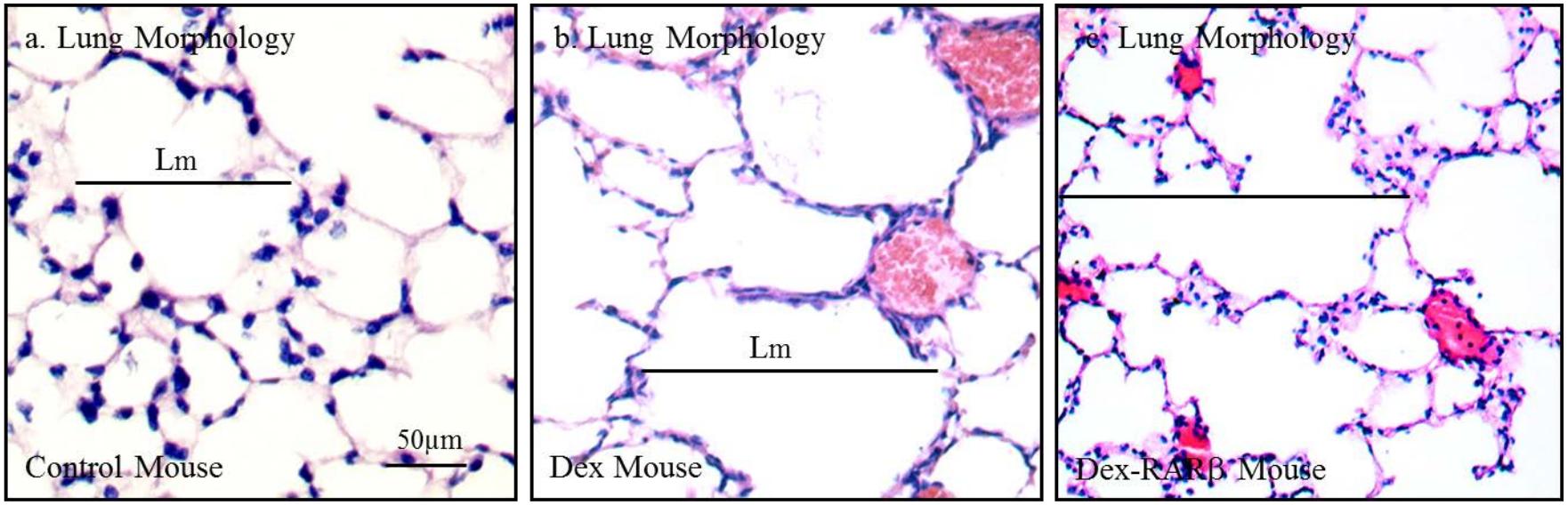

Fig 4.1.4a-c: Lm. Morphology of postnatal mouse lung (H&E staining). Representative transverse sections show typical structure of alveoli seen in postnatal dex-RARβ mouse lungs (c) compared with dexamethasone (b) and control mouse lungs (a). Black lines represent where the measurements for Lm are calculated. Cell walls of dex-RARβ lungs (c) appeared thin, similar to dexamethasone lungs (b). Scale bar = 50µm

The next retinoid we examined for regenerative efficacy was RARγ in the dexamethasone group. Morphological, the induction of this retinoid showed pockets of “normal” looking alveoli structures, but in general, this group presented enlarge areas of airspace (Fig 4.1.5c). Their cell walls appeared thinner than dexamethasone lungs (Fig 4.1.5b). Their alveolar septa were short. Like RARα, there was a reduction in vascularisation in this group, again suggesting a positive response to inflammation. The average Lm value of this group was 87.1 ± 2.0μm (Table 4.1.1). In terms of a recovery response, this group delivered a percentage value of 28%, which was 21% lower than dex-tRA and was 72% less than the control group. When comparing this group with previous retinoid treatments, there was a difference of 16% with dex-RARα and a 9% difference with dex-RARβ (Table 4.1.1). In spite of this increase in Lm values in comparison with the dex-tRA group, dex-RARγ Lm values were closest to dex-RARα (82.8 ± 3.μm). Statistically, when comparing dex-RARγ Lm’s with the dexamethasone group, a significant value of $p<0.05$ was achieved indicating that this group was not as efficient was tRA. Sa values of dex-RARγ lung group gave an average value of 338.7 ± 24.0cm², a difference of 6.7cm² in comparison with dex-tRA treatment.

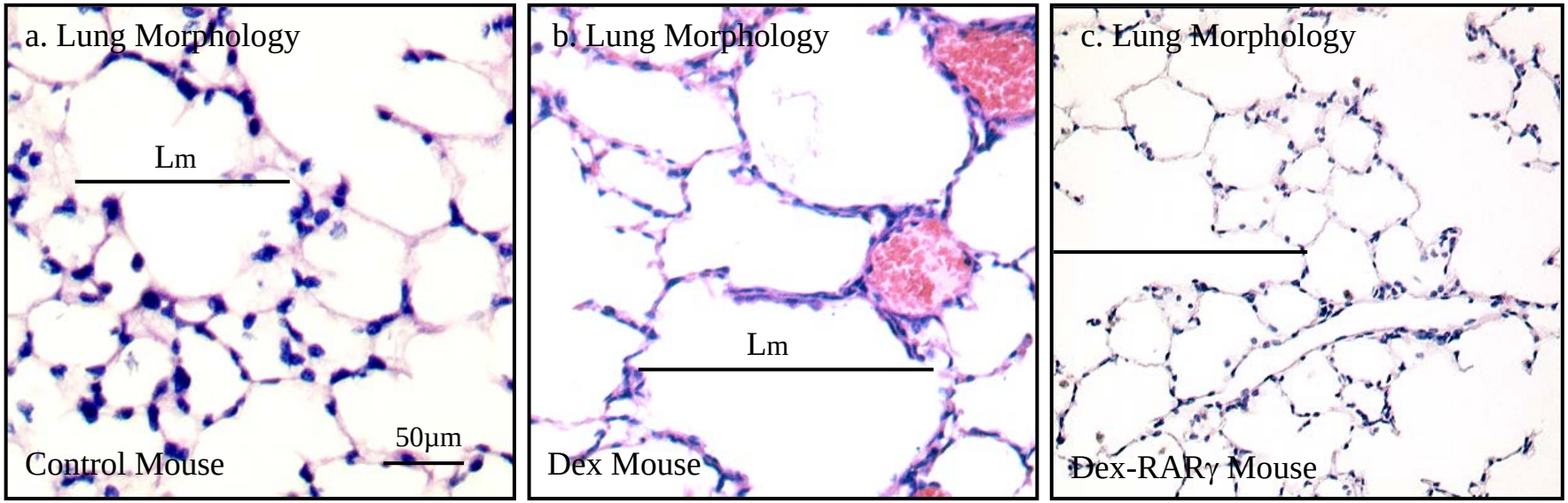

Fig 4.1.5a-c: Lm. Morphology of postnatal mouse lung (H&E staining). Representative transverse sections show typical structure of alveoli seen in postnatal dex-RARγ mouse lungs (c) compared with dexamethasone (b) and control mouse lungs (a). Black lines represent where the measurements for Lm are calculated. Cell walls of dex-RARγ lungs (c) appeared thin, similar to dexamethasone lungs (b). There were pockets of alveolar population where structure and size appeared normal. Scale bar = 50µm

The final retinoid we examined was a combination of ligands for RXR and for the purpose of this thesis was termed pan-RXR. On the surface, this retinoid group showed morphological features similar to control lungs (Fig 4.1.6a and c). Alveolar airspace and cell wall thickness appeared “normal” and identical to control lungs. There appeared to be a slight increase in vascularisation in comparison to control lungs; alveolar septae were slightly pointed. ATII cells were inconsistent in structure. Some appeared oval in shape whilst others squamous-like, but both structures were small in appearance (Fig 4.1.6b and c). The average L_m value of this group was 94.0 ± 3.0µm (Table 4.1.1). This value was the highest amongst the other retinoid treatments and was identical to dexamethasone average values; 94.3 ± 2.1µm. This was the second time where morphological features contradicted L_m values. Differences in Lm values were as follows; 12µm with dex-tRA, 11.2 µm with dex-RARα, 9.4 µm with dex-RARβ and 6.94 µm with dex-RARγ. A non-statistical value of 0.7460 was given when compared with dexamethasone lungs, deeming this retinoid treatment the worst of the retinoid groups in terms of a regenerative response. In comparison to dex-tRA treatment, there was a significant difference of 0.0079, confirming dex-panRXR lack of response to dexamethasone destruction. When comparing this groups Sa (242.6 ± 20.9cm²) with dex-tRA, it gave a an enormous difference of 102 µm. When evaluating dex-panRXR’s data with the previous retinoid treatments, the differences were as follows; 59.6cm² with dex-RARα, 32.2cm² with dex-RARβ and finally 91.1cm² with dex-RARγ.

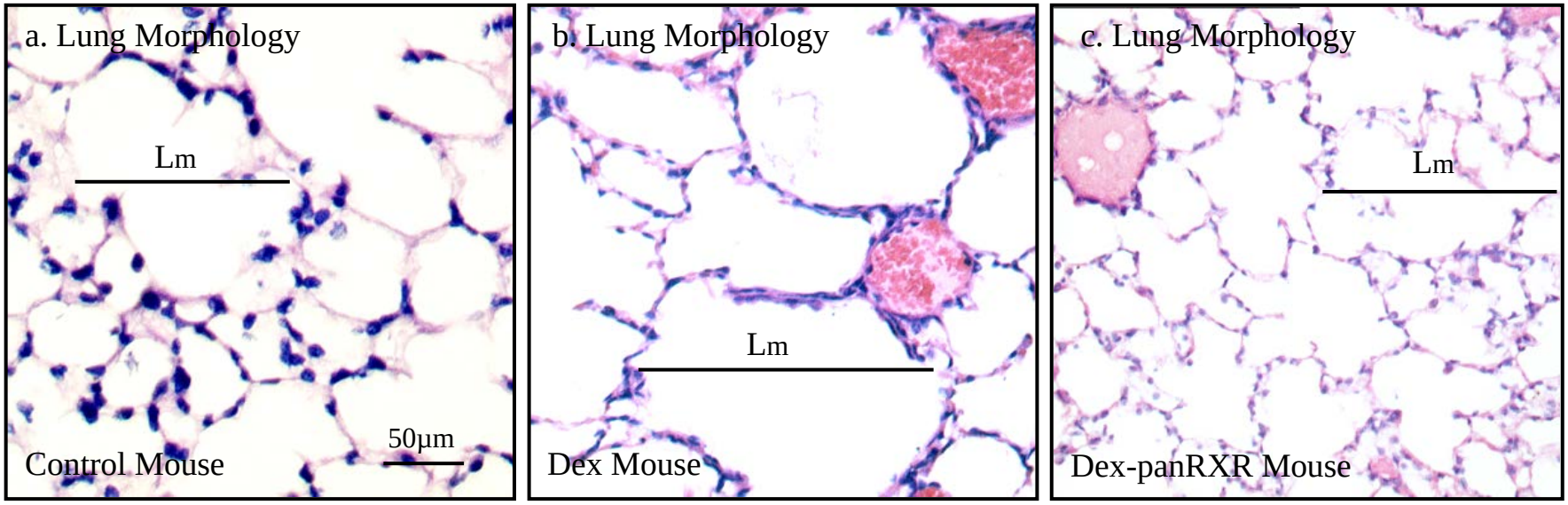

Fig 4.1.6a-c: Lm. Morphology of postnatal mouse lung (H&E staining). Representative transverse sections show typical structure of alveoli seen in postnatal dex-panRXR mouse lungs (c) compared with dexamethasone (b) and control mouse lungs (a). Black lines represent where the measurements for Lm are calculated. Cell walls of dex-panRXR lungs (c) appeared thin, similar to dexamethasone lungs (b). There was a slight increase in vascularisation compared with control lungs (a). Scale bar = 50µm

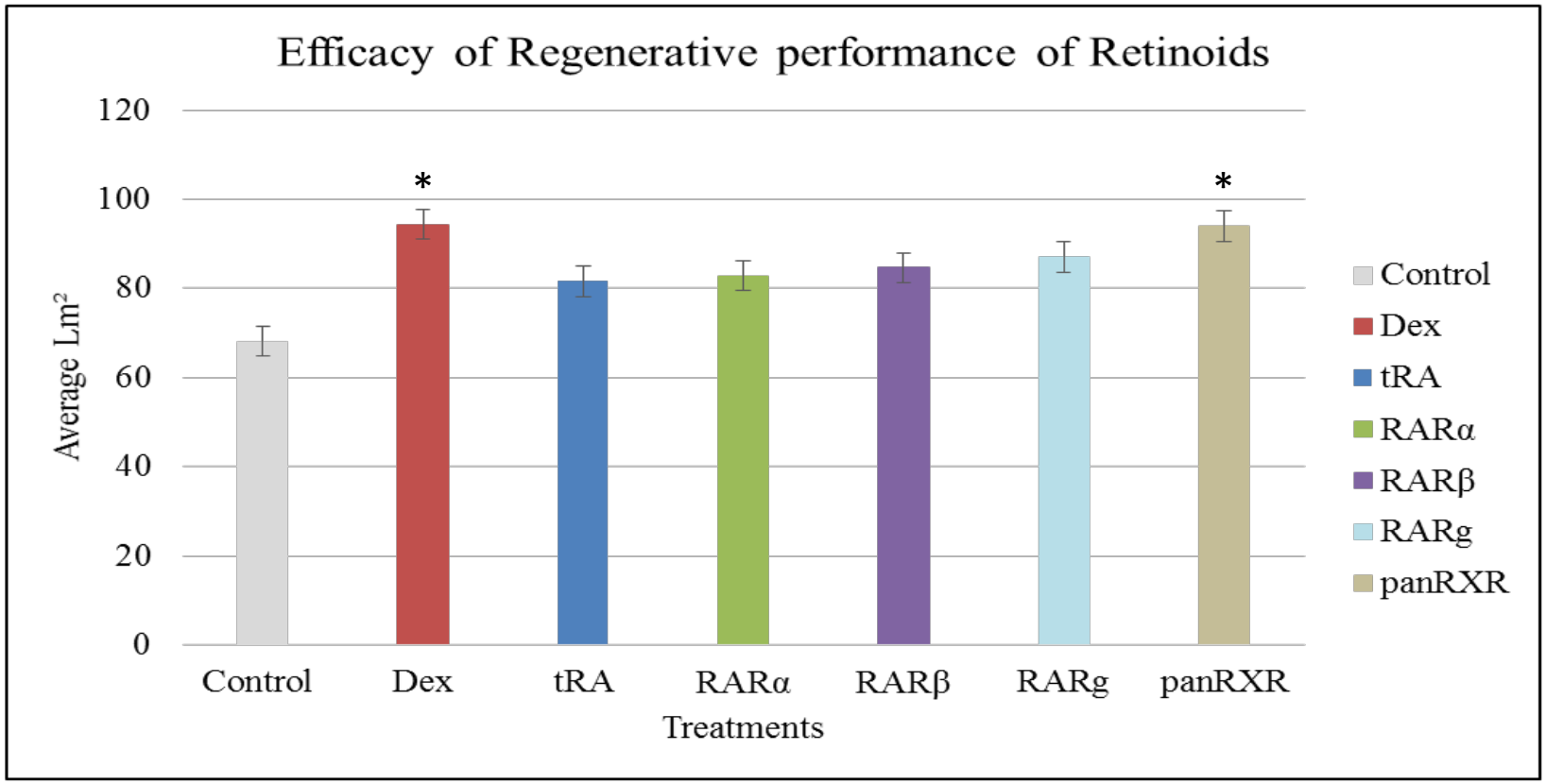

Fig 4.1.7: Regenerative Efficacy of retinoid response to dexamethasone effect on airway morphology. Graph shows a summary of how receptor agonists of retinoids respond individually to damaged lungs. RARα showed similar response to dexamethasone as the retinoid, tRA. This was followed by RARβ, RARγ. Pan-RXR showed no recovery response.

Table 4.1.1: Measurements of Control, Dex, tRA, RARα, RARβ, RARγ and panRXR Lungs

	No	Mean Lm (μm)	Mean Recovery (%)	Mean Volume (cm^2)	Mean Sa (cm^2)	Mean Sa/volume Ratio
Control	10	68.1±0.9	100	0.740 ± 0.015	434.7 ± 7.5	589.0 ± 7.8
Dex	21	94.3±2.1	0	0.717 ± 0.028	305.3 ± 10.5	428.5 ± 9.5
tRA	8	81.6±2.0	49	0.702 ± 0.038	345.4 ± 18.1	492.7 ± 13.5
RARα	15	82.8±2.8	44	0.633 ± 0.050	302.2 ± 18.5	490.7 ± 16.1
RARβ	15	84.6±2.3	37	0.579 ± 0.035	274.8 ± 13.8	479.9 ± 11.6
RARγ	7	87.06±2.3	28	0.734 ± 0.047	338.7 ± 24.0	465.8 ± 14.4
panRXR	8	94.0±2.5	0	0.561 ± 0.048	242.6 ± 20.9	436.3 ± 13.7

ANOVA test: Data are presented as ± SEM. Table shows a summary of the difference in airspace, Sa/volume and recovery. Dex = Dexamethasone, tRA = all-trans-Retinoic acid, RARα = Retinoic Acid Receptor-Alpha, RARβ = Retinoic Acid Receptor-Beta, RARγ = Retinoic Acid Receptor Gamma, panRXR = Retinoid X receptor, Lm = Mean chord length, Sa = Surface area

4.1.4: DISCUSSION. It is evident that the growth and maintenance of postnatal lungs are affected by the action of vitamin A's active form tRA along with its receptor molecules, the retinoids, RAR and RXR. Numerous studies involving a variety of animal models have shown vitamin A to have both proliferative and apoptotic characteristics, an essential requirement for the normal structure and functioning of healthy lungs.

tRA has been shown to rescue the lungs of mice and rats from dexamethasone-induced induced inhibition of postnatal lung formation (Massaro and Massaro 1996, Hind and Maden 2004, Stinchcombe and Maden 2008, Seifart, Muyal et al. 2011). In addition, several experimental studies in rats and mice suggest that regeneration of lost alveolar can be achieved by tRA in the elastase and cigarette-induced emphysema (Massaro and Massaro 1997, Belloni, Garvin et al. 2000, Seifart, Muyal et al. 2011). However, a number of studies have failed to demonstrate a beneficial effect of tRA in other emphysematous models and indeed any beneficial effects in human patients (March, Barr et al. 2002, Lucey, Goldstein et al. 2003, Fujita, Ye et al. 2004, March, Cossey et al. 2004, Yoshimatsu, Nan-ya et al. 2009, Seifart, Muyal et al. 2011). Clinical trials that have taken place with patients of the disease have so far been marginal (Mao, Goldin et al. 2002, Mao, Tashkin et al. 2003, Roth, Connett et al. 2006, Frankenberger, Heimbeck et al. 2009, Seifart, Muyal et al. 2011). The reasons for these discrepancies are still unclear, although potential mechanisms underlying the beneficial effects of tRA in dexamethasone-induced impairment of alveolarization have been suggested. Only little is known about the molecular mechanisms contributing to its potential regenerative effects in the smoking or elastase-induced models of emphysema (Liebeskind, Srinivasan et al. 2000, Mao,

Goldin et al. 2002, Mao, Tashkin et al. 2003, Maden 2006, Massaro and Massaro 2006, Roth, Connett et al. 2006, Zhao, Wang et al. 2006, Seifart, Muyal et al. 2011).

There have also been studies where naturally occurring and synthetic retinoids have been used to demonstrate their regenerative abilities in animal models of respiratory diseases, namely emphysema, thereby signifying their potential use as therapeutic agents for such airway diseases. However, like most primary studies, there have been concerns surrounding dosing and the nature of retinoid(s) used as administration can lead to numerous side effects including skin irritation, lipid and bone toxicity and severe interference with normal embryonic development (teratogenic). To counteract these effects, receptor agonists have been developed with the intention of only activating subsets of genes in the retinoid pathway (Martin, Bernardon et al. 1992, Nagpal and Chandraratna 2000, Dawson and Zhang 2002, Farmer, Zhi et al. 2003, Piu, Gauthier et al. 2005, Maden 2006). The results have led to specific retinoid agonists having effects on for example, promoting growth and cell death, gene induction and epithelia metaplasia; thus enabling researchers to decipher the many different characteristics of these retinoids and closely scrutinise the each retinoid agonist plays in the respiratory system (Sun, Yue et al. 2000, Pendino, Dudognon et al. 2003, Maden 2006).

Focusing on the dexamethasone postnatal mouse model, we first demonstrated the lost of alveolar structure using this steroid and then examined the regenerative therapeutic efficacies of tRA and a range of four naturally occurring and synthetic

retinoid receptor agonists (RARs and RXRs) after administration via histology morphology and morphometric parameters (the measurement of alveolar airspace).

As previously discussed in the introduction of this chapter and chapter 1 (1.5), dexamethasone permanently impairs the process of alveolarization, the critical stage of alveolar formation, which in healthy conditions forms the classical honeycomb features seen in mature lungs. By impairing this process, abnormally enlarged alveolar airspaces is generated with extremely thin alveolar cell walls, thus creating increased alveolar airspace (Lm) values and a much reduced gas exchanging Sa and also impairs ATII cell replication (Massaro and Massaro 1986, Chytil 1996, Hind and Maden 2004). Dexamethasone is used to treat infant with lung disorders such as bronchopulmonary dysplasia (BPD), but only as a short-term treatment, as long term will lead to the clinical features mentioned above. To amend this architectural damage, Hind and Maden showed this model to be highly responsive to tRA because new septae was induced, resulting in an increase in the number of alveoli, a decrease in Lm values and an increase in gas exchanging Sa.

Individually, we examined retinoid agonist receptor molecule regenerative efficacies after the administration of dexamethasone-induced emphysema and compared results with tRA treatment of these dexamethasone lungs in this study. The first retinoid we examined was RARα. This retinoid group of lungs showed increase in septal thickness and a significant decrease in alveolar airspace when compared with dexamethasone-treated lungs. An increase in ATII cell population was indicative of alveolar regeneration. Comparing RARα-treatment with tRA-treatment showed

strong correlation between the two treatments, indicating that RARα may be considered potentially as a good candidate for alveolar regeneration. Overall calculated values for this group showed that this retinoid was only slightly higher than tRA; 82.8 ± 3.0μm (RARα) and 81.6 ± 2.0μm (tRA) for Lms. The average difference was only 1.2μm and there was only a 5% less recovery rate in this retinoid group compared with tRA treated groups with a p value difference of 0.6667, confirming RARα's strong regenerative properties. Perhaps, it could be argued, because of its high performance, if this retinoid had continued slightly longer, it could be argued that it may have shown even better results than tRA, as RARα is known to enhance alveolar septation and is required for normal morphogenesis (Mahadeva and Shapiro 2002). The next two retinoids examined were RARβ and RARγ. These two retinoid groups showed similar structural performances. Both alveolar cell walls were extremely thin and in fact resembled structural features seen in dexamethasone treated lungs. Both groups showed areas of ATII cells located in the alveolar epithelia to be squamous-like, a feature also seen in the dexamethasone group, but unlike the RARγ group, RARβ group showed a slight increase in vascularisation, again similar to the dexamethasone group. Previous studies have shown that RARβ has positive regenerative properties in injurious lungs. Studies have also been shown to be required for morphogenic formation as well as gene expression in the form of *Fgf10* (fibroblast growth factor) and bud formation in the lung (Desai, Malpel et al. 2004, Desai, Chen et al. 2006). It was demonstrated when endogenous RARβ is deactivated, *Fgf10* expression is disrupted which is a significant feature as *Fgf10* possess broad mitogenic and cell survival and is involved in a variety of biological processes including embryonic development, cell growth, morphogenesis, tissue repair, tumour growth and

invasion. However, this was not established in this study. It may be that in the dexamethasone postnatal mouse model, RARβ needs to be administered at a much earlier stage of injury. RARβ non-responsiveness to regeneration has also been shown in clinical trials where genetically induced emphysematous patients showed no obvious lung function improvement, but showed mild side effects, which included skin irritation. On the other hand, RARγ is supposedly expressed in the skin and lung of adult mice. It is highly expressed in lung fibroblasts immediately after birth, so it is surprising that this retinoid showed a poor performance in our postnatal model (Kastner, Krust et al. 1990, McGowan, Harvey et al. 1995, McGowan, Jackson et al. 2000). Morphometric analysis calculating the overall performance of the retinoids used was slightly contradictory with RARγ agonist when comparing its regenerative efficacy with tRA. For example even though morphologically, RARγ gave some good structural alveolar features, morphometric analysis showed that this retinoid was in fact the worst of the RAR agonists in terms of recovery. Values were almost identical to dexamethasone-induced emphysematous lungs and indeed gave a non-significant p value of $p=0.7460$, confirming that in this instance, RARγ is perhaps not required during the postnatal period of lung development. panRXR was the only retinoid that did not give a recovery response. In previous regenerative studies of emphysematous animal models RXR showed recovery, but using individual compounds of receptor molecules. However, in our model, maybe because we used a combination of the receptors counteracting with each other, regeneration was not accomplished. The order of efficacies was as follows: tRA> RARα>RARβ. Because the other two compounds were not statistically significant, we did not include them in the efficacies, even though they showed some degree of recovery.

Data obtained from this study implies that the mechanism of alveolar regeneration by retinoids involves the action of an RAR and not a RXR agonist, although, as just mentioned, panRXR made it difficult to determine its true efficacy. Nevertheless, the regenerative action of RAR is a common finding in retinoid biology because a RAR/RXR heterodimer is receptor dimer. On the basis of this study, it would seem that the agonist RARα is the active heterodimer component in alveolar regeneration. The fact that RARα has been shown to play a role in alveolus formation, not in the immediate postnatal period, but between the ages of 14 to 50 days supports our agonist data because this is the exact period we administered retinoid treatment. So we can conclude that RARα is involved in the regenerative response to injurious lungs.

4.1.5: SUMMARY. The lung is a major tissue for storage of retinol as retinyl esters (Liu 2005, O'Byrne, Wongsiriroj et al. 2005, Gudas 2012). Exogenous RA can stimulate retinol uptake and storage in the lung. From the data and previous studies presented, we can conclude that the three RAR molecules play distinct roles in the mouse lung. RARα is need for alveoli population in the postnatal period whereas RARβ functions to inhibit alveoli formation, thus creating a balanced equilibrium and that RARγ may produce new alveoli, but at a much slower rate. RARγ is known to be required for the first few weeks after birth for the completion of lung formation (Gudas 2012). It may be that a combined effect of RARα and RARβ may be required to not only produce new alveoli, but to also prevent overproduction of new growth.

CHAPTER 4.2

THE REGENERATIVE EFFECT OF RETINOIDS ON AIRWAY PROGENITOR CELLS IN POSTNATAL MOUSE LUNGS

OBJECTIVES: Respiratory diseases trigger airway progenitor cells such as Clara cells of the bronchiole airway epithelium and ATII cells of the alveolar airway epithelium. The present study was designed to examine Clara and ATII cells in response to injury and repair of the airway epithelium following the administration of retinoid ligands after induced emphysematous created by dexamethasone in postnatal mouse lungs. By using biomarkers CC10 (for Clara cells), SP-C (for ATII cells), PCNA (for proliferation) and TTF-1 (for Nkx2.1 transcription marker), we were able to demonstrate changes in expression and function of these airway progenitor cells. Expression of ATII cells in dexamethasone-treated lungs showed a much reduced cell population in the alveolar epithelium when compared with control animals. It suggested that this was due to cell differentiation of ATII cells to ATI cells. There was no immediate change in the level of expression with Clara cells in bronchiole airway epithelium of dexamethasone animals when compared with control animals, however, there were distinctive cellular changes of these progenitor cells. The structure of these cells appeared squamous-like and irregular in shape alone the bronchiole epithelium and there was no real evidence of Clara cell proliferation, suggesting functional alteration. Both dex-tRA and dex-RARα lung groups showed a reduction in Clara cell population but Clara cell proliferation indicating bronchiole epithelial regeneration. Dex-RARβ, RARγ and pan-RXR lung groups showed basal proliferation, but not Clara cell proliferation suggesting repair and not regeneration.

4.2.1: INTRODUCTION. The main function of progenitor cells in the airway epithelium is homeostasis and repair if defected walls of the airways (Giangreco, Arwert et al. 2009). These defects may be due to trauma brought on by mechanical malfunction that requires replacement of epithelial tissue. In airway diseases such as asthma, COPD and CF, the airway epithelium is damaged and subsequently requires repair or regeneration to take place (Hajj, Baranek et al. 2007, Coraux, Roux et al. 2008, Burgess, Hu et al. 2009, Tzortzaki and Siafakas 2009).

Repair or regeneration of damaged airway epithelia by disease is a major field where progenitor cells have an essential role. However, in contrast to some tissues, such as gut, haematopoietic system, skin and hair follicles, adult progenitor cells in the lung are ill defined. Adult progenitor cells are not immortal and show decreasing telomere length with increasing age of the individual, which may result in the inability to repair/regenerate the onset of damage created by chronic disease. The possibility to manipulate adult progenitor cells later in life could potentially improve chronic airways diseases such as emphysema.

The wall of the trachea and bronchi in the normal mammalian airway consist of a surface epithelium with ciliated, brush, basal and secretory cells that rest on a basement membrane, followed by layers of connective tissue and smooth muscle and finally cartilage, the bronchiole lack cartilage. The epithelium in the major bronchi and proximal bronchioles is a ciliated, pseudostratified epithelium with ciliated columnar cells as the predominant cell type; in the distal bronchioles the epithelium consists of a single cell layer. The ciliated columnar cells reach the basal lamina

with slender basal processes the function of the brush cells of the airway epithelium is not completely known (Shebani, Shahana et al. 2005). These cells lack cilia and may be involved in detoxification, act as a sensor for airway surface liquid or may have a chemoreceptor function. Various secretory cells (goblet cells in the trachea, bronchi and proximal bronchioles, Clara cells in the bronchioles and intrapulmonary airways) comprise around 20% of the bronchial and nasal epithelium and produce airway secretion (fluid and mucus). In addition, Clara cells secreted Clara cell secretory protein, an immuno-regulatory protein (Synder, Drobniewski et al. 2009). The walls of the alveoli consist of squamous ATI and surfactant-producing ATII cells (Fig 4.2.1).

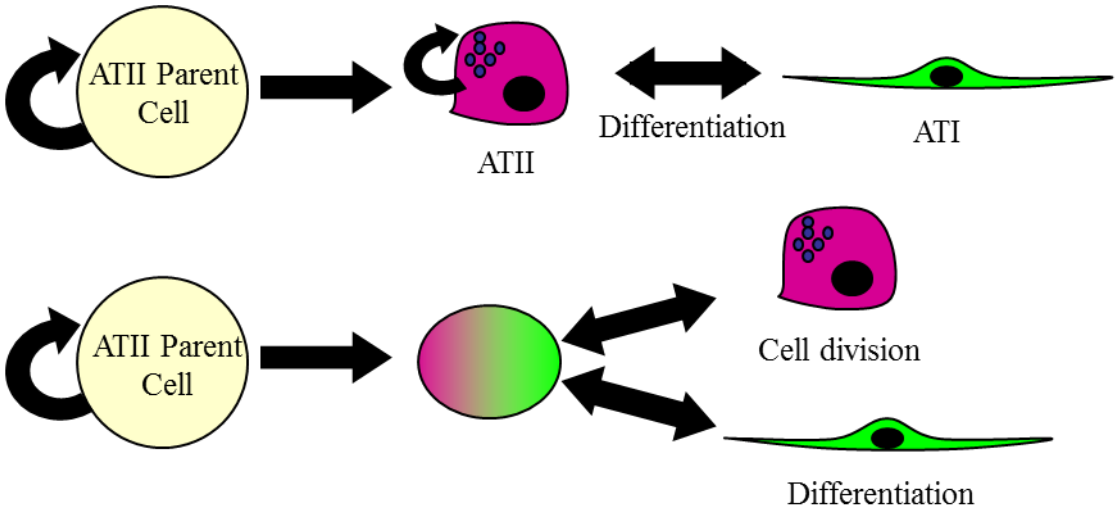

Fig 4.2.1: Cellular mechanism of regeneration of ATII cells and differentiation of ATI cells from parent cell (ATII)

Under healthy conditions, there is to a certain degree cell turnover in the airways. Tissues with a relatively slow cell turnover, use self-renewal of differentiated cells as a strategy for tissue regeneration (Rawlins and Hogan. 2006). In addition, damage to the airway epithelium occurs frequently e.g. due to the components of smoke, air pollution or bacterial and viral infections, either from direct effects of these microorganisms or due to an inflammatory reaction that follows infection. Normally in the case of small lesions, airway epithelium possesses repair mechanisms that restore normal structure. However, if the damage becomes chronic, e.g. in COPD patients, the repair process is more extensive and is called airway remodelling (Folli, Descalzi et al. 2008). Airway regeneration appears to be controlled by bronchial epithelial and endothelial cells of the bronchial vasculature (Zani, Cordischi et al. 2008). Of these two cell types, the bronchial epithelial cells have received by far the most attention whereas little is known about the role of endothelial cells (Rawlins, Hogan. 2008, Rawlins, Okubo et al. 2009). ATII cell injury impairs normal production, secretion and recycling of pulmonary surfactant. This in turn leads to loss of surface tension-reducing phospholipids, alveolar instability and collapse (Lewis, Jobe. 1993, Greene, Wright et al. 1999).

Additional work suggest that Clara cells and macrophage signalling is critical for the response to injury as measured by production of epithelial and macrophage specific chemokines and cytokines. This process is initiated by secretion of paracrine signalling molecules from macrophages such as tumour necrosis factor (TNF)-α that target the airway epithelium as an intermediate signalling cell type required for production of neutrophilic chemotatic factors (Elizur, Adair-Kirk et al. 2008). Collectively, these results indicate that the distal airway and respiratory epithelial

cells play a prominent role in providing important immuno-regulatory properties essential for the regulation of innate immunity.

Among the many functions of Clara cells is secretion of Clara cell secretory protein and is the predominant protein product of emphysema whose expression precipitously declines in COPD. Data suggest that epithelial secretion is compromised as a result of epithelial remodelling and Clara cell injury in the setting of COPD. Through precise differentiation of cell types organized within functional zones, relative to anatomical location, the epithelium plays an active role in host defence. Collectively, this data indicates that airway epithelial specification, effective repair and maintenance are critical for lung homeostasis. Defects to these processes or significant epithelial remodelling may contribute to COPD through altered epithelial function and perturbations to the host defence properties of the lung.

Understanding the aetiology and progression of COPD is complicated due to the complex network of cellular cross-talk present in the lung. Defective airway epithelial repair has been proposed as an early event in the initiation of COPD and chronic defects in reparative capacity and cellular composition may further contribute to disease progression and susceptibility (Holgate, Holloway et al. 2004). This implies that normal functions for facultative progenitor and stem cells are compromised. Evidence suggesting that this may be the case comes from analysis of biomarkers of epithelial remodelling that have been used to follow severity and progression of lung disease.

4.2.2: AIM. tRA plays a key role in the development and differentiation of many cell types within the lung. Transcriptional factors mediate the majority of RA functions and other transcriptionally active RA metabolites. (Gudas. 2012). The mechanisms that control levels and activities of enzymes that produce metabolites such as RA from retinol are not quite fully understood (Linke, Dawson et al. 2005, D'Ambrosio, Clugston et al. 2011, Kumar and Duester. 2011, Lee, Kim et al. 2011). Importantly, levels of endogenous retinoids are altered in many different diseases contributing to the pathophysiology. In many cell types, RA promotes cell differentiation and proliferation.

The aim of this study was to establish lung regeneration from retinoid ligands through progenitor cell growth. We examined ATII and Clara cell population with view to proliferative and transcriptional activity of these two cell populations. The basic behaviour of progenitor cells, their molecular regulators and how each is impacted by disease would provide novel insights into the pathogenesis of disease and reveal new therapeutic targets that may allow modifications in the outcome of disease. Furthermore, understanding the effects of abrogating epithelial repair on modulation on the epithelial function will be equally important, especially as it relates to the initiation and progression of COPD.

4.2.3: RESULTS. IF Single labelling detection of Progenitor Cells: To investigate whether retinoid ligands promote repair/regeneration in dexamethasone-induced emphysema, we ran a series of experiments examining the airway progenitor cells, Clara (bronchi tree) and ATII (alveolar) cell expression along with transcriptional and proliferation activity in postnatal mouse lungs. Table 4.2.1 summarises the number of Clara cells expressed as the percentage mean of bronchiole airway epithelial cells. In control lungs the Clara cell marker, CC10 was demonstrated in the bronchiole epithelium. Expression was achieved via IF labelling of paraffin embedded sections measuring 5µm in thickness. CC10-positive Clara cells in these lungs displayed the classic tall columnar dome-shape with a "cap" on top of each cell (Fig 4.2.2a). Cells were not shown to be expressed throughout the bronchiole epithelium, there were patchy areas of staining in each bronchiole confirming no cellular expression. Bronchioles in dexamethasone-treated lungs demonstrated an increased level of expression of CC10-positive Clara cells in comparison with control lungs (Fig 4.2.2b). However, structural differences between CC10-positive Clara cells in dexamethasone and control in the bronchiole epithelium were evident. Cells in this group did not display the classical tall columnar dome shape with a cap on top; instead some presented themselves as flat (shown in Fig 4.2.2b) whereas others appeared dysplastic. It is conceivable that an increase in CC10-positive Clara cells would be detected in this group of lungs as Clara cells play an important defensive role and contribute to the degradation of mucus production by the upper airways. Statistical analysis revealed an average cell count of 253 ± 4.4 in dexamethasone lung groups compared with control lung groups which gave an average cell count of 189.4 ± 13.2 (Figs 4.2.3 and 4.2.4).

We compared the response of Clara cells to the induction of tRA after dexamethasone lung injury (Fig 4.2.2c). The morphology of these cells in this group appeared tall columnar similar to that observed in control lung groups. There were a few differences in this group in comparison to control groups. Cells were thinner of suggesting less secretory CC10 protein. Cells were tightly packed along the epithelia, but there was a reduction in the number of positive cells. The average cell count was 134.8 ± 7.3. There was a significant difference between dex-tRA and dexamethasone lung groups; the p value was <0.01. Its classical domed cape shape situated on the tops of cells had also returned.

We next examined the effect of RARα ligand on Clara cells. Morphology of these cells in this group showed a bigger improvement in this group. These cells were not only tall columnar and domed shaped with a cap on the apical surface of each cell, but more importantly, they appeared more filled out than positive CC10-Clara cells in dex-tRA lung groups (Fig 4.2.2d). Cell population in dex-RARα group had also improved in comparison to dex-tRA lungs. They had risen from 134.8 ± 7.3 to 146.3 ± 7.0. Again, the significant value in the difference of Clara cell response in dexamethasone and dex-RARα was $p<0.01$.

The morphological appearance of CC10-positive Clara cells in dex-RARβ lungs appeared damaged (Fig 4.2.2e). These cells looked severely dysplastic and had lost their classical morphological appearance; they were squamous-like in shape. There were areas along the bronchiole epithelium where CC10 expression was intermittent. Cell counts in this group of lungs were difficult to calculate because it was difficult

to view them as individual cells. Nevertheless, the average cell count for this lung group was 128.5 ± 2.0 compared to dexamethasone (253 ± 4.0) and control (189.4 ± 13.2). This group expressed fewer cells than dex-tRA lung group (134.8 ± 7, Fig 4.2.4). Again, when this group of regenerative lungs were compared with dexamethasone, there was a significant difference, $p < 0.01$ (Table 4.2.1).

Next, we examined the effects of RARγ ligand. The morphology of CC10-positive Clara cells showed much improvement in cell morphology when compared with Clara cells of dexamethasone lungs (Fig 4.2.2f). Cell morphology was similar to dex-RARα, but again like CC10-positive Clara cells in dex-RARβ lungs there was not much improvement in the level of expression and therefore showed no significant difference between the two groups, but was between dex-RARγ (123. 9 ± 6) and dexamethasone (253 ± 4) groups. The p value was also <0.01.

The final retinoid to be examine for Clara cell response to regeneration was panRXR. Clara cells that were positive for CC10 were tall columnar in morphology and were domed-shaped (Fig 4.2.2g). Cells in this group had a low expression. Cells that were present were evenly spaced with areas of absent cells (127.4 ± 8, Fig 4.2.3 and 4.2.4). Compared with other retinoid ligands, this number proved to be less than any of the other retinoid ligands used for regeneration of dexamethasone lungs. Again, this group gave an overall significant difference of $p<0.01$.

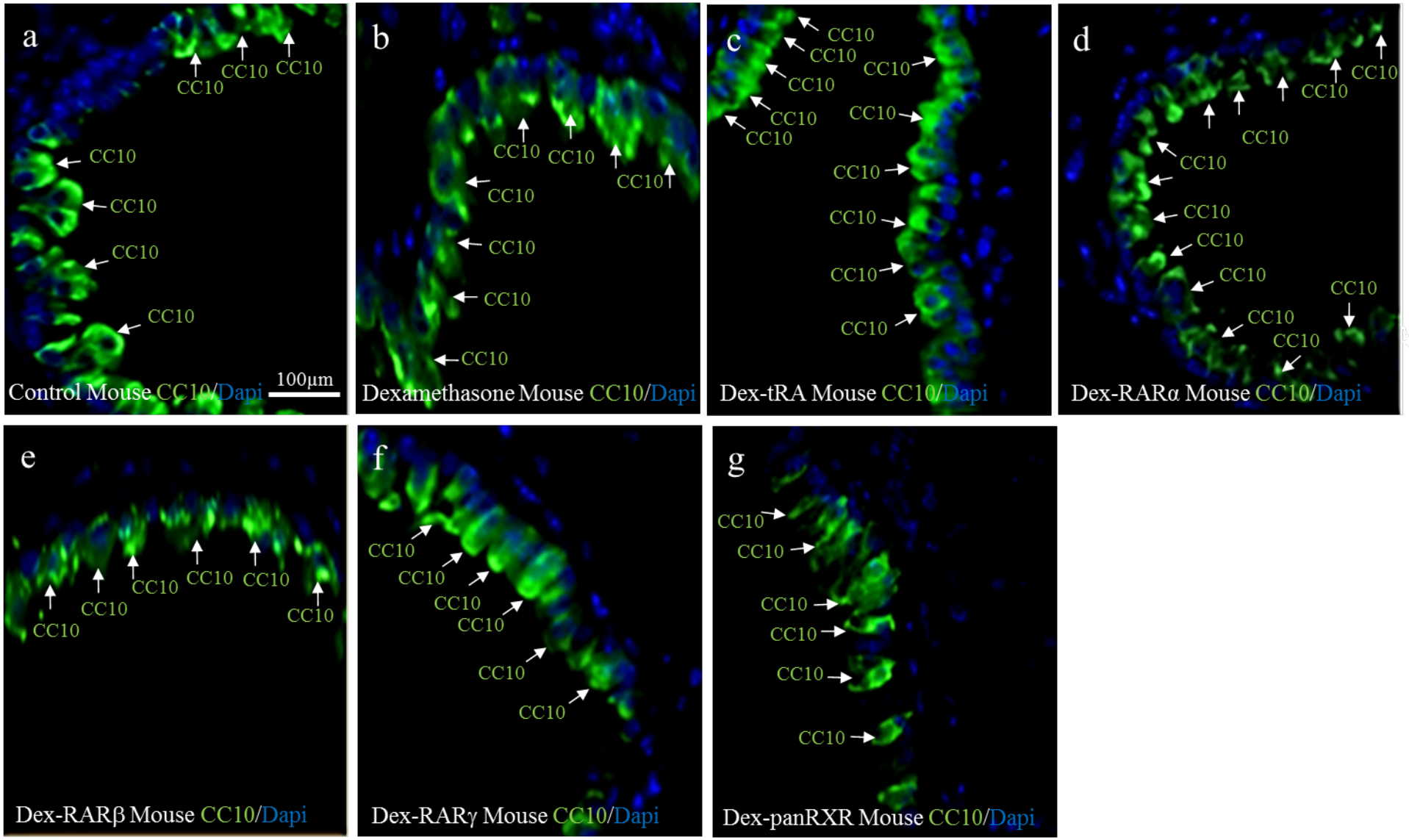

Fig 4.2.2: IF labelling of secretory CC10 in Clara cells of control (a) and dexamethasone (b) and retinoid treated lungs (c-g) in the bronchiole airway epithelium. Clara cells in bronchiole epithelium of dexamethasone lung groups distinctly showed cell alteration, but more CC10-positive cells, suggesting a subset of cells and an increase in cellular defence. tRA, RARα, γ and panRXR showed similarity in cellular structure in comparison with control lungs (a). RARβ (e) showed no cellular difference from dexamethasone lungs.

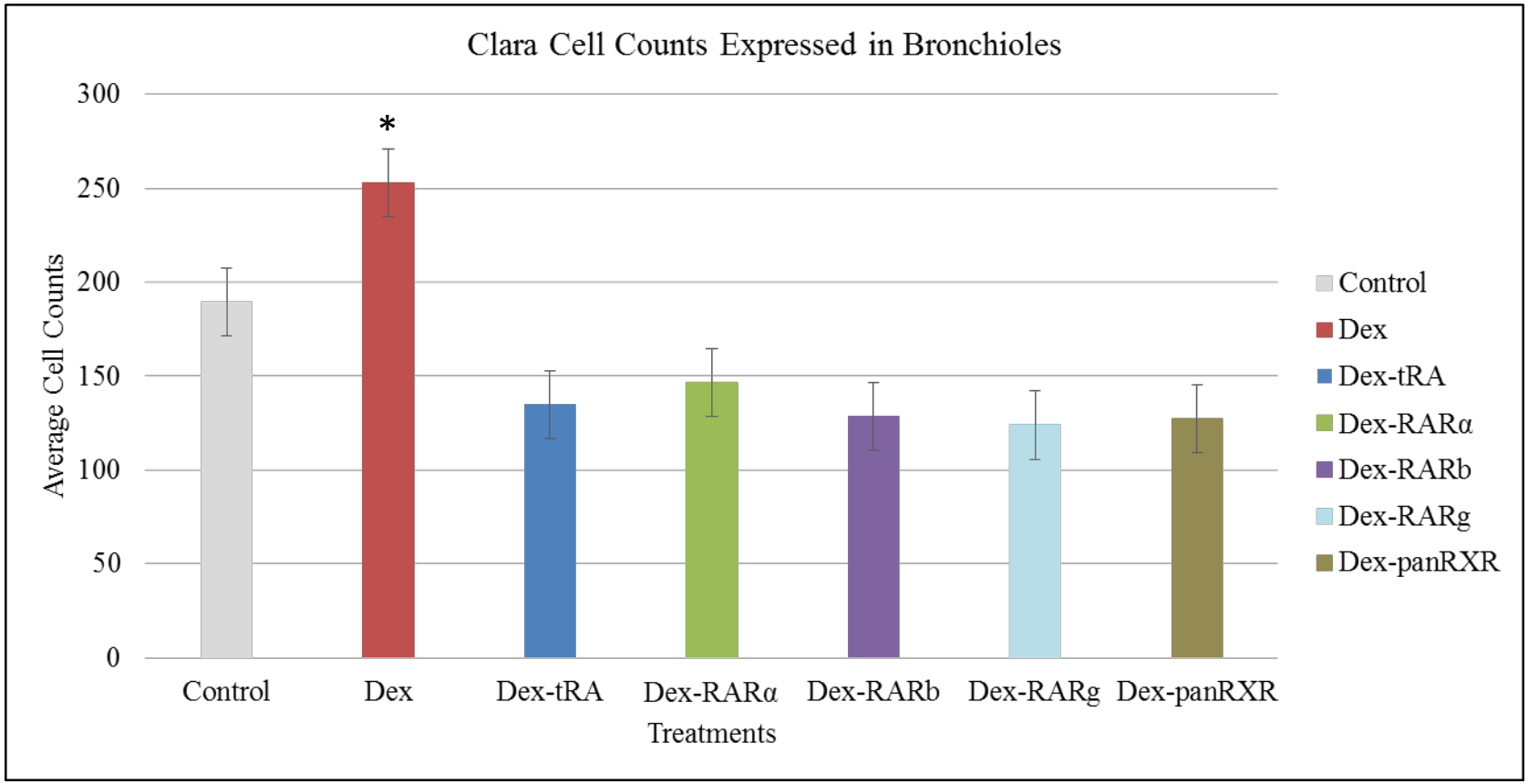

Fig 4.2.3: Graph shows mean Cell counts of CC10-positive Clara cells (±) SEM in the bronchiole epithelium of dexamethasone and various retinoid ligand treatments. Statistical analysis was done by one-way ANOVA and Bonferroni post-test. P<0.01 * represents P=0.004. Average cell counts show an increase in CC10-positive Clara cells in dexamethasone lung groups suggesting a response to injury/bronchiole repair with reduction of cells in retinoid ligands. The reduction of cells in retinoid ligand lung groups could suggest the ligands response to inflammation promoted by dexamethasone.

Table 4.2.1: One-way ANOVA followed by Bonferroni post-correction test for CC10-positive Clara cells in postnatal mouse lungs

	Control	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	208.40	253.00	134.75	146.267	128.467	123.857	127.375
SD	41.81	20.15	20.44	28.96	7.51	15.03	21.49
SEM	13.22	4.40	7.23	7.48	1.94	5.68	7.6
N	10	21	8	15	15	7	8

Dexamethasone treated lung showed a significant increase of CC10 levels of secretion compared to retinoid treated lungs. This could signify a response to inflammation.

SP-C/ATII positive cells were restricted to the alveolar airway epithelium. Expression demonstrated in control lungs showed SP-C/ATII cells surrounding alveoli nuclei. These cells were cuboidal in shape and fairly uniform (Fig 4.2.4a). Most cells in this lung group appeared enlarged and granulated possibly indicating cell proliferation. There was no indication of SP-C staining demonstrating ATI cells in these lungs. Following dexamethasone treatment, SP-C/ATII cells were less cuboidal, much thinner in structure. These cells did show granulation, but also cells that were squamous-like suggesting proliferation and differentiation taking place along the alveolar epithelium (Fig 4.2.4b). The average cell counts for control and dexamethasone groups were as follows; 312.6 ± 13.6 and 282.9 ± 5 respectively (Fig 4.2.5, Table 4.2.2).

We examined the effect of tRA on the cellular structure and population of ATII cells. tRA proved to be most efficient in the reversal of dexamethasone effects on this cell population. The average cell count for this group was 336.6 ± 21 (Fig 4.2.5). The difference between dexamethasone and dex-tRA lung groups gave a p value of 0.01. Morphologically, SP-C/ATII cells in this group were similar to those observed in control lungs, cuboidal, large and granular (Fig 4.2.4c). There were some cells that had lost their morphology structure. These cells were squamous-like, so could have been ATII cells, there did not appear to be as much of these cells present in this group as there were in the dexamethasone group.

Dex-RARα lung group had a higher SP-C/ATII population than dex-tRA and control groups but cellular structure was irregular and did not appear granular (Fig 4.2.4d).

Cells in this group were much smaller and quite spacious. Cell numbers for this group were as follows; 372.7 ± 9.9 (Fig 4.2.5, Table 4.2.2). SPC-ATII cells presented in dex-RARβ lung group had a low population. Morphological appearance was similar to cells shown in dex-RARα, small, spacious and non-granular. They did not appear cuboidal. Average cell count for this group was 183.1 ± 7 (Fig 4.2.5, Table 4.2.2). There appeared to be a mixture of ATII and ATI cells in dex-RARγ with no appearance of granulation (Fig 4.2.4f). Finally, we examined the efficacy of pan-RXR. The morphology of ATII cells were mostly squamous-like in appearance and were small. These cells were very similar to cells observed in RARβ and RARγ lung groups (Fig 4.2.4g). Cell counts for this group was 127.4 ± 8 (Fig 4.2.5, Table 4.2.2). Comparing these three retinoid ligand with tRA showed that their regenerative performance was less efficient and RARα was proven to be the overall best ligand in terms of this mouse model. The overall outcome of this study showed ATII to respond to dex-RARα readily than the other retinoid ligands. The other three ligands may have had a lower ATII population, but from observations of IF staining suggests ATII differentiation to ATI cells in response to epithelial repair of alveoli.

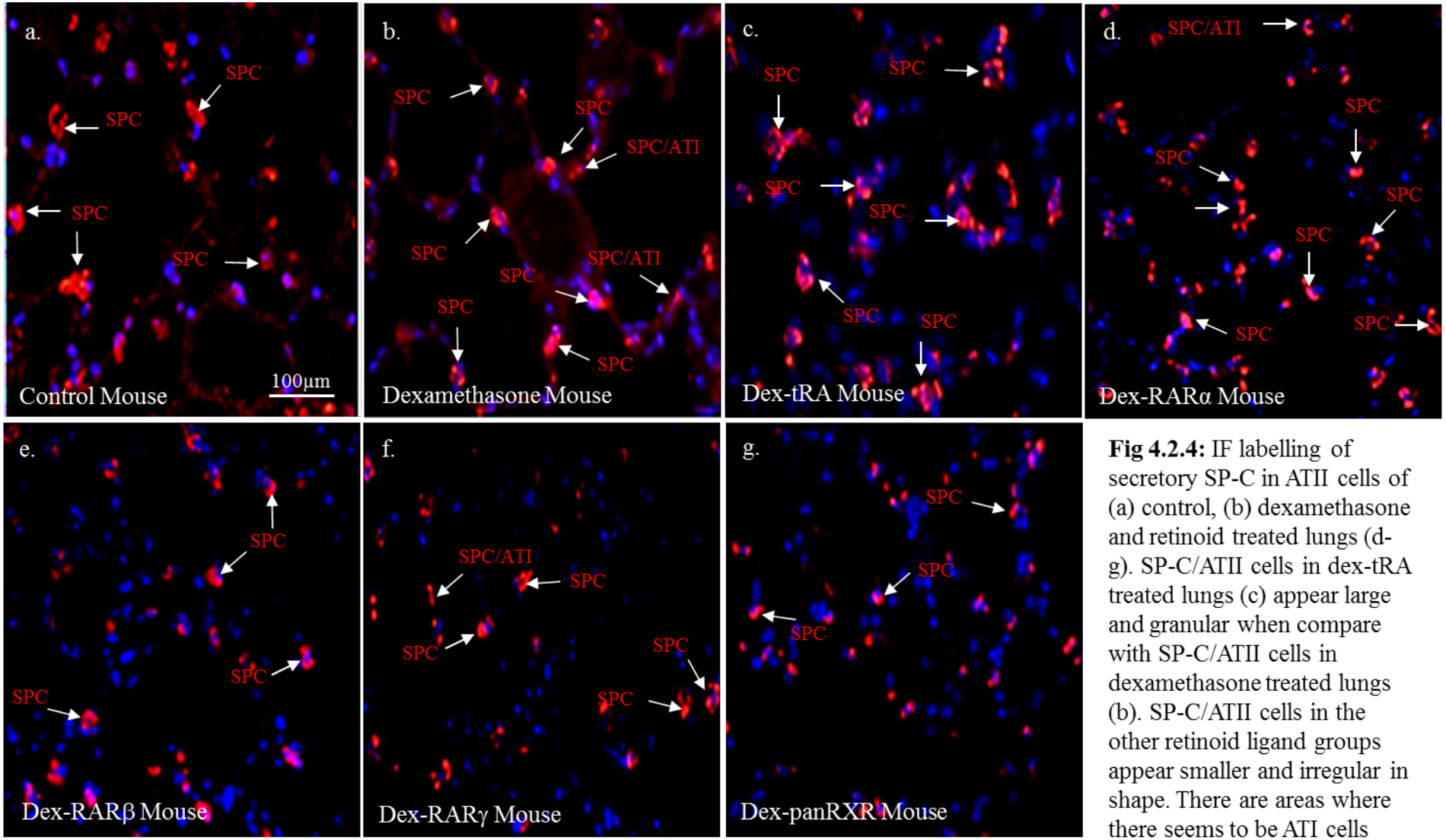

Fig 4.2.4: IF labelling of secretory SP-C in ATII cells of (a) control, (b) dexamethasone and retinoid treated lungs (d-g). SP-C/ATII cells in dex-tRA treated lungs (c) appear large and granular when compare with SP-C/ATII cells in dexamethasone treated lungs (b). SP-C/ATII cells in the other retinoid ligand groups appear smaller and irregular in shape. There are areas where there seems to be ATI cells

This possibly could be due to cell differentiation and repair/regeneration of alveolar epithelia. There was a greater reduced number of cell population in dex-panRXR lung groups.

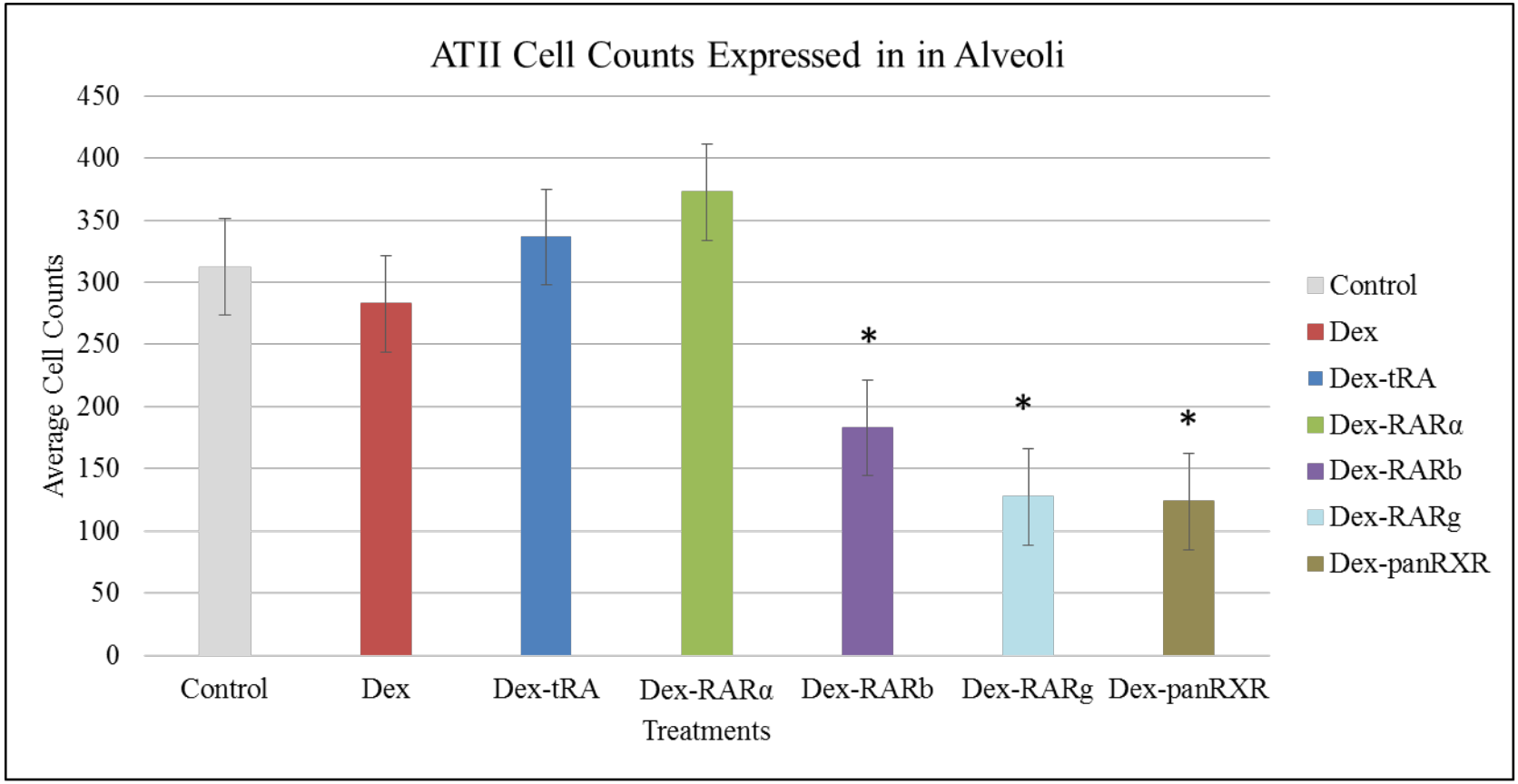

Fig 4.2.5: SP-C cell counts in the alveolar airway epithelium. Graph shows AVONA statistical analysis of SP-C/ATII of dexamethasone and various retinoid ligands treated lungs. Dex-RARβ, dexRARγ and dex-panRXR had the lowest numbers of ATII cells. This could suggest cellular differentiation of ATII cells into ATI cells.

Table 4.2.2: One-way ANOVA followed by Bonferroni post-correction test for SP-C/ATII cells in Postnatal mouse lungs.

	Control	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	312.6	282.9	336.6	372.7	183.1	127.4	123.9
SD	42.9	21.2	59.8	38.3	26.8	22.1	40.2
SEM	13.6	5.0	21.1	9.9	7.0	15.1	14.2
N	10	21	8	15	15	7	8

Dexamethasone treated lungs shows a significant decrease in expression of SP-C protein and therefore a decrease in the number and structural abnormality of ATII cells secreting surfactant. This suggests damage has occurred to the alveolar airway epithelium. Equally, tRA and RARα lung groups show an increase in the expression of SP-C and therefore an increase in the number of ATII cells and a change in morphology, signifying a recovery of the epithelium in these lung groups

IHC detection of Nkx2.1 using the antibody TTF-1 showed similar pattern of expression observed with SP-C/ATII cells in the alveolar and bronchiole airway epithelia in control lungs (Fig 4.2.6 and 4.2.7). TTF-1 staining was strongly expressed in alveolar epithelium. Expression in dexamethasone treated lungs was also strong, but there was a reduced number of cell population. The average cell count for control and dexamethasone were 320.5 ± 10 and 279.7 ± 3 respectively. This correlation was deemed statistically different; >0.01 (Fig 4.2.8, Table 4.2.3). This reduction of TTF-1 staining coincided with expression of SP-C/ATII cells in dexamethasone lungs. Dex-tRA lungs showed much improvement in TTF-1 expression. Expression was strong. Pattern of staining was uniform in expression in alveolar epithelium and indicated epithelial regeneration. This expression was extremely similar to that observed in control lungs (Fig 4.2.7c). Expression in this group of lungs suggests tRA aids the promotion of alveolar epithelial repair/regeneration. Average cell counts for TTF-1 in this group was 346.7 ± 13.3 confirming upregulation of Nkx2.1 transcriptional activity in alveolar epithelia. Expression of TTF-1 in dex-RARα showed to be spacious and strongly stained. Again expression was similar to TTF-1 expression in control lungs, cells were cuboidal and nuclear. Calculation confirmed an upregulation of TTF-1 staining in this group when compared with tRA, control and dexamethasone lung groups (Fig 4.2.6d). The average cell counts of TTF-1 staining in this group was 388.9 ± 9.4 (Fig 4.2.8, Table 4.2.3). Expression of TTF-1 in dex-RARβ was low, as was SP-C/ATII staining. There were very few cells in the region of the alveolar epithelium, any present in the location was quite hard to detect. Average cell counts for TTF-1 was 113.1 ± 6.0. The p value for this group was >0.01 making this difference significant.

Dex-RARγ also gave low detection, but were much higher than dex-RARβ. Cellular expression was also easier to detect in this group of lungs, but cells presented in this group of lungs were sparse (Fig 4.2.6f). Average cell counts for this group was 205.7 ± 11 (Fig 4.2.8, Table 4.2.3). Finally we examined expression pattern of TTF-1 in dex-panRXR. Staining was similar to dex-RARα. Positive cells were cuboidal and nuclear in morphology and strong in expression (Fig 4.2.6g). There were areas where the staining was sparse, but nevertheless, expression was high. Cell counts for this group was 236 ± 13 (Fig 4.2.8, Table 4.2.3).

TTF-1 expression in the bronchiole epithelium was strong in control lungs. Cells were tall columnar in morphology (Fig 4.2.7a). There were obvious differences in the expression of TTF-1 staining in dexamethasone. Firstly, there was a reduction of TTF-1/Nkx2.1 staining and the morphology of these cells were squamous-like along the bronchiole epithelium (Fig 4.2.7b). The average cell count of these cells were 266.5 ± 9 in control lungs and 115.3 ± 1.1 in dexamethasone lungs (Fig 4.2.8, Table 4.2.3). These values too gave a significance of >0.01. The level of expression observed in dexamethasone lungs suggest that SP-C/ATII cells are dependent on Nkx2.1 signalling, but not CC10/Clara cells in this particular mouse model. TTF-1 staining in dex-tRA bronchiole was weak and appeared to have altered pattern of expression from that of control. TTF-1 appeared to be outlining goblet cells lining the epithelia. Cellular expression of TTF-1 in this group of lungs was less than control and dexamethasone. Expression seem to coincide with CC10/Clara cell staining where there was a much reduced number of cells in control and dexamethasone groups. This suggests tRA may not be involved in bronchiole repair/regeneration. The average cell count of TTF-1 was 196.6 ± 5 (Fig 4.2.8, Table

4.2.3). TTF-1 expression in the bronchioles of dex-RARα was columnar and strongly stained. There were areas where there was no staining, same patterning as control lungs (Fig 4.2.6d). This suggests that RARα is also vital for the repair/regeneration process in bronchiole epithelium and therefore repair and regeneration of the lung as a whole. Average cell counts for this group of lungs was as follows; 188.1 ± 16 (Fig 4.2.8, Table 4.2.3). Staining in the bronchioles of dex-RARβ lungs was strong, squamous-like in appearance and very similar to dexamethasone lung group (Fig 4.2.7e). Expression in the bronchiole was much better than that seen in alveolar epithelium in this lung group. Average counts was 117.6 ± 3.0. Staining was irregular in dex-RARγ. Pattern of staining was not tall columnar, expression of TTF-1 was shown to be apical (Fig 4.2.7f). Average cell count was 95.0 ± 2 (Fig 4.2.8, Table 4.2.3). Mean was, in comparison with dexamethasone less, suggesting that RARγ may not have a major role in bronchiole repair/regeneration. Expression of TTF-1 was similar to dex-RXR expression (Fig 4.2.7g) suggesting that these ligands are not exclusively involved in bronchiole epithelial repair/regeneration. The average cell count for this group was 108.4 ± 2.

Overall in sequence, dex-RARα followed by dex-tRA gave the most positive results in promoting TTF-1 transcription activation for regeneration to occur. This set of results coincided with previous data obtained in this thesis where both derivatives performed best morphologically and morphometrically (Chapter 4.1).

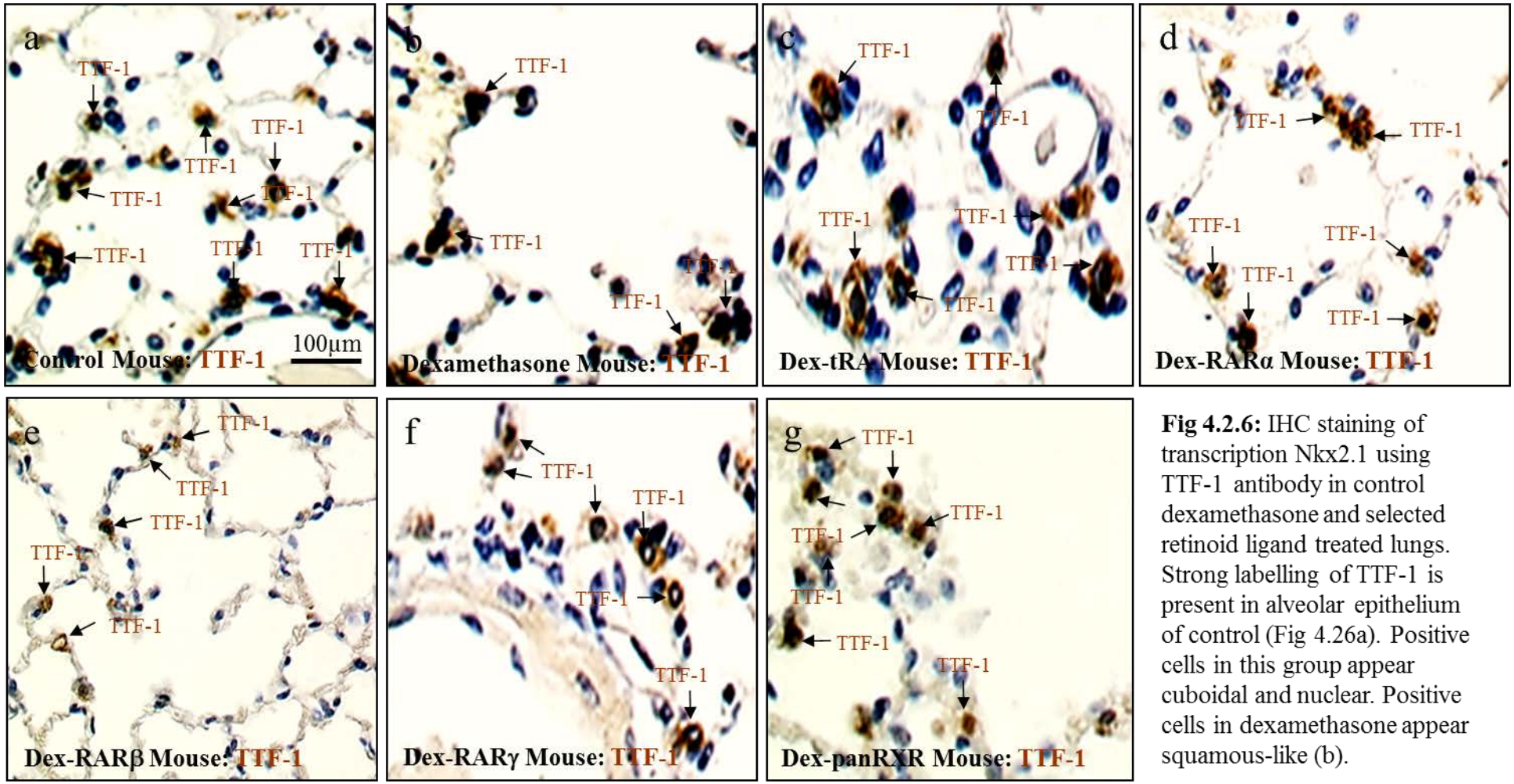

Fig 4.2.6: IHC staining of transcription Nkx2.1 using TTF-1 antibody in control dexamethasone and selected retinoid ligand treated lungs. Strong labelling of TTF-1 is present in alveolar epithelium of control (Fig 4.26a). Positive cells in this group appear cuboidal and nuclear. Positive cells in dexamethasone appear squamous-like (b). tRA, RARα and panRXR treated lungs showed an increase in transcriptional activity (c and d). Expression had decreased in RARβ lungs (e) suggesting that RARβ may not be involved in alveolar airway repair/regeneration.

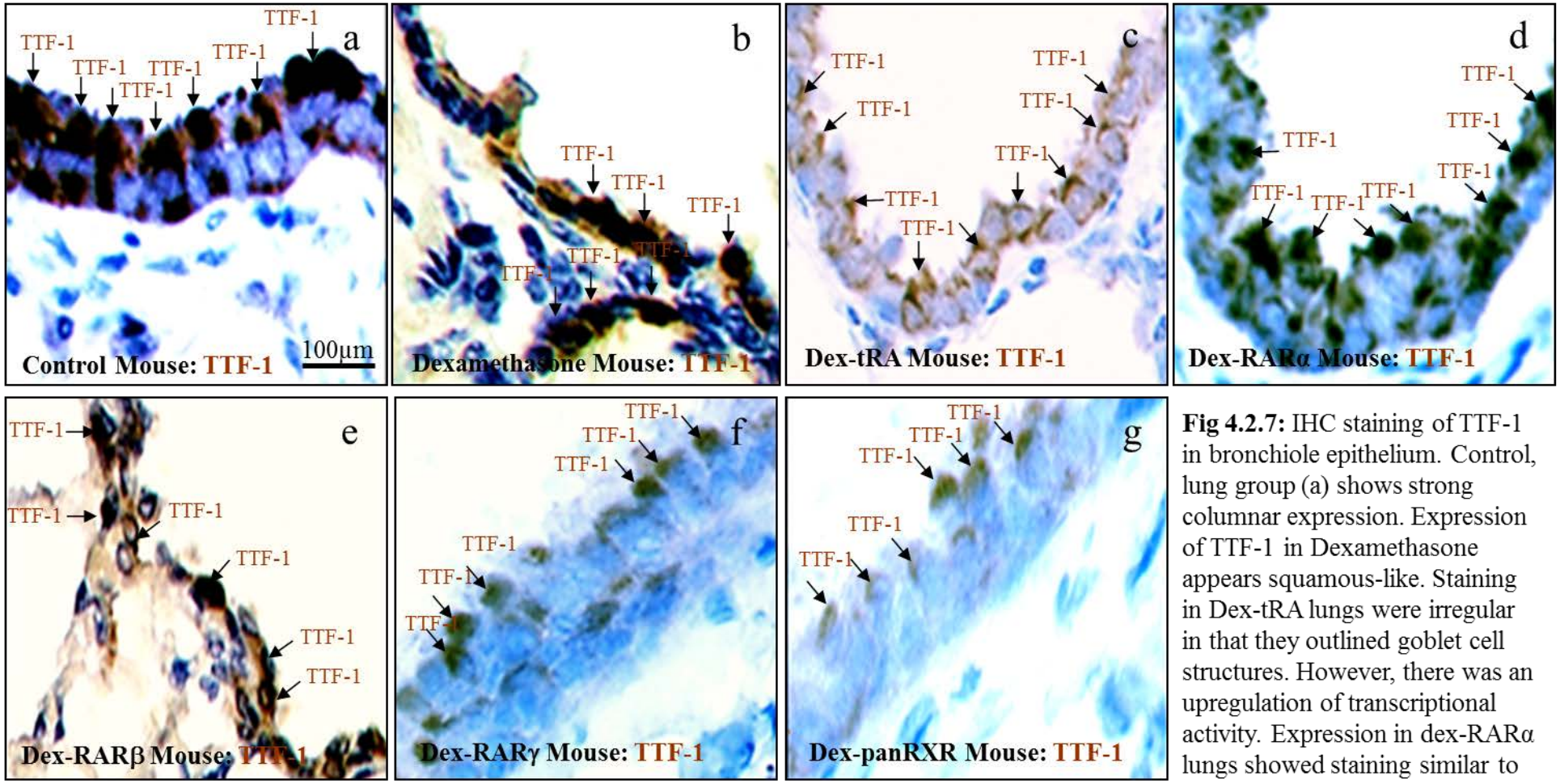

Fig 4.2.7: IHC staining of TTF-1 in bronchiole epithelium. Control, lung group (a) shows strong columnar expression. Expression of TTF-1 in Dexamethasone appears squamous-like. Staining in Dex-tRA lungs were irregular in that they outlined goblet cell structures. However, there was an upregulation of transcriptional activity. Expression in dex-RARα lungs showed staining similar to TTF-1 expression in control lungs. This group also showed upregulation of transcriptional activity. Squamous-like features of TTF-1 staining was also demonstrated in dex-RARβ and gave similar cell counts as dexamethasone lung groups. Pattern of staining could suggest that this ligand has no reversal of change from dexamethasone. There was downregulation of TTF-1 in dex-RARγ and dex-panRXR. These three ligands suggest that they have no major role in bronchiole repair/regeneration in this mouse model.

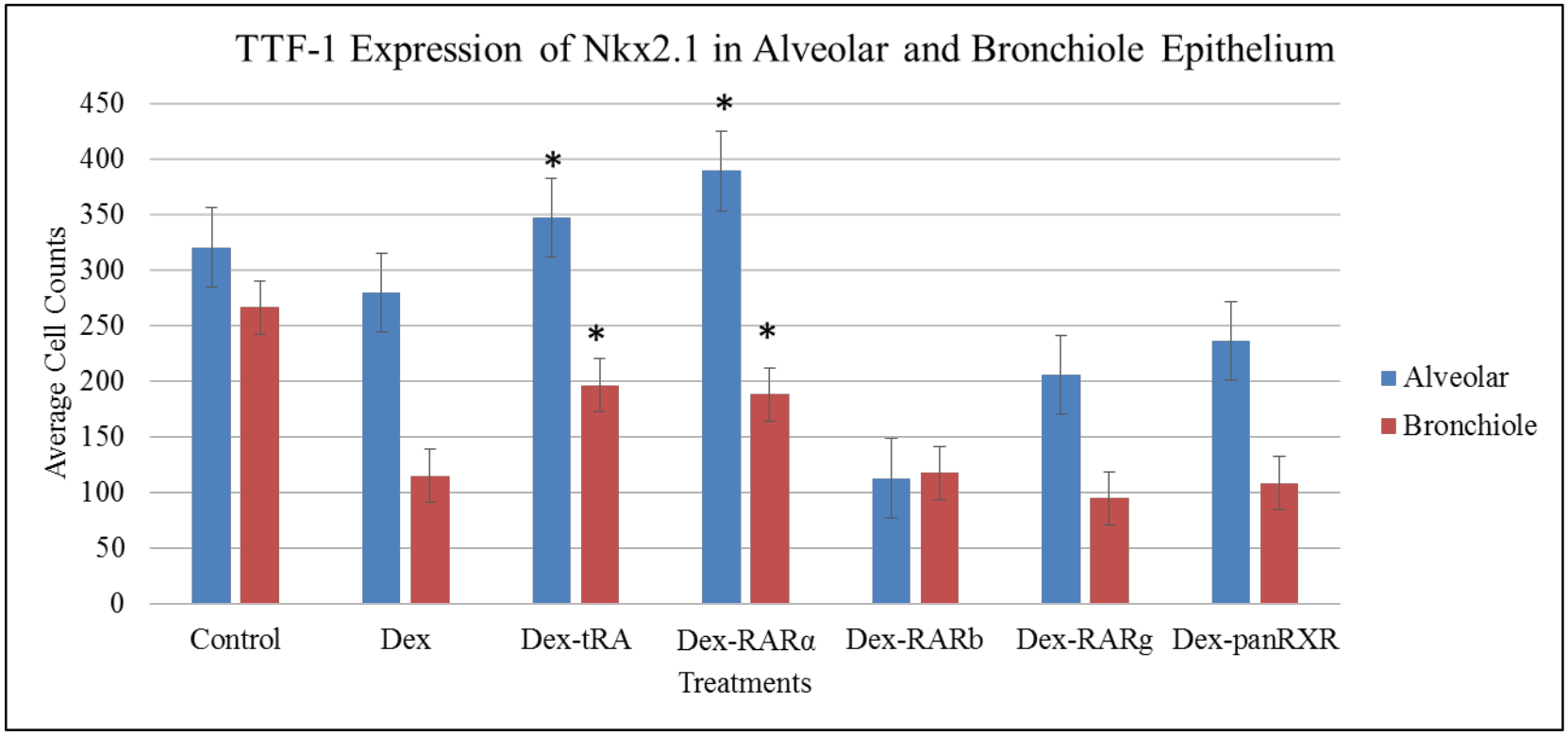

Fig 4.2.8: Summary of TTF-1 expression for Nkx2.1 in alveolar and bronchiole epithelia. Graph shows that the overall RA derivatives that appear to be involved in epithelial repair/regeneration are tRA and RARα.

Table 4.2.3: One-way ANOVA followed by Bonferroni post-correction test for TTF-1 Expression in parenchyma

Alveolar	**Control**	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	320.5	279.7	346.7	388.9	113.1	205.7	236.0
SD	32.09	14.42	51.64	36.32	9.77	31.14	35.47
SEM	10.15	3.15	13.33	9.38	2.52	11.77	12.54
Bronchiole	**Control**	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	266.5	115.3	196.6	188.1	117.6	95.0	108.4
SD	28.91	49.72	19.83	60.12	24.91	6.47	6.46
SEM	9.14	10.85	5.12	15.52	6.43	2.44	2.28
N	10	21	8	15	15	7	8

Dexamethasone shows expression confirms its effect on epithelial cells in response to injury. There is a correction of TTF-1 and SPC expression. Where there is a reduction of TTF-1, there is also a reduction in SP-C and vice versa when TTF-1 is upregulated in alveolar epithelium suggesting TTF-1 regulates ATII secretory surfactant postnatally. However, where there was a reduction of TTF-1 there was an upregulation of CC10/Clara cell in the bronchiole epithelium suggesting that these cells are independent of transcriptional regulation postnatally in this model of injury.

IF double labelling of CC10 and SP-C with PCNA: Following the demonstration of Clara and ATII cells during and after lung injury, we investigated cellular proliferation as part of bronchiole/alveolar repair/regeneration of the epithelium. As noted, Clara cells generally proliferate at a slow rate. In order to establish cellular proliferation, we employed PCNA which is known to be important for DNA synthesis and DNA repair. We first examined Clara cell proliferation.

We noted that there was high proliferative activity within the bronchiole epithelia of control lungs. Amongst this, basal cell proliferation was extremely high, but no presence of Clara cell proliferation (Fig 4.2.9a). There was also no Clara cell proliferation observed in dexamethasone lungs. There were also fewer proliferating basal cells in this lung group (Fig 4.2.9b). Nevertheless, we carried out basal cell counts. The average counts for these cells was 85 ± 3 in control and 49 ± 1 (Fig 4.2.10, Table 4.2.4). When examining dex-tRA, we observed Clara cell proliferation within the bronchiole epithelia (Fig 4.2.9c). There was strong colocalisation between CC10/PCNA, however, this feature was not prominent throughout the epithelium. Expression suggested damage to the bronchiole epithelia caused by dexamethasone was going through the process of repair/regeneration in this lung group. The average cell counts for this group was 70 ± 5 (Fig 4.2.10). Because this cell count was for Clara cell proliferation and not basal cell, we did not compare statistical analysis with dexamethasone and control groups. The same proliferative results seen in dex-tRA lungs were seen in dex-RARα (Fig 4.2.9d). Just as was observed in dex-tRA, not all Clara cells underwent proliferation, but like in dex-tRA lung group, we concluded that this showed a degree of bronchiole epithelial repair/regeneration. Again, because control and dexamethasone lung groups did not show Clara cell

proliferation we did not compare dex-RARα with either group. We did however calculate a comparison between dex-tRA and dex-RARα (Fig 4.2.10). The average cell count for this group was 80 ± 4 compared with dex-tRA (70 ± 5). The p value between these two groups was <0.05 deeming this non-significant. Dex-RARβ showed basal cell but not Clara cell proliferation (Fig 4.2.9e). The average cell count for this group was 50 ± 1. Comparing this average with dexamethasone showed no significant difference between the two groups (Fig 4.2.10). Again there was no presence of Clara cell proliferation in dex-RARγ lungs and very little basal cell proliferation (Fig 4.2.9f). The average cell count for this group was 80 ± 2 (Fig 4.2.10). Finally dex-panRXR were examined. Like dex-RARβ and dex-RARγ, this group did not show any Clara cell proliferation, but did show basal cell proliferation. This was seen along the bronchiole epithelium (Fig 4.2.9g). The average cell count was similar to control lungs; 83 ± 3 (Fig 4.2.10).

Both basal and Clara cells are involved in bronchial repair and regeneration. From the results of the cell counts (Fig 4.2.10) we concluded that dex-RARβ gave the least repair/regenerative response to epithelial damage because basal cell numbers were almost identical to basal cell numbers in dexamethasone.

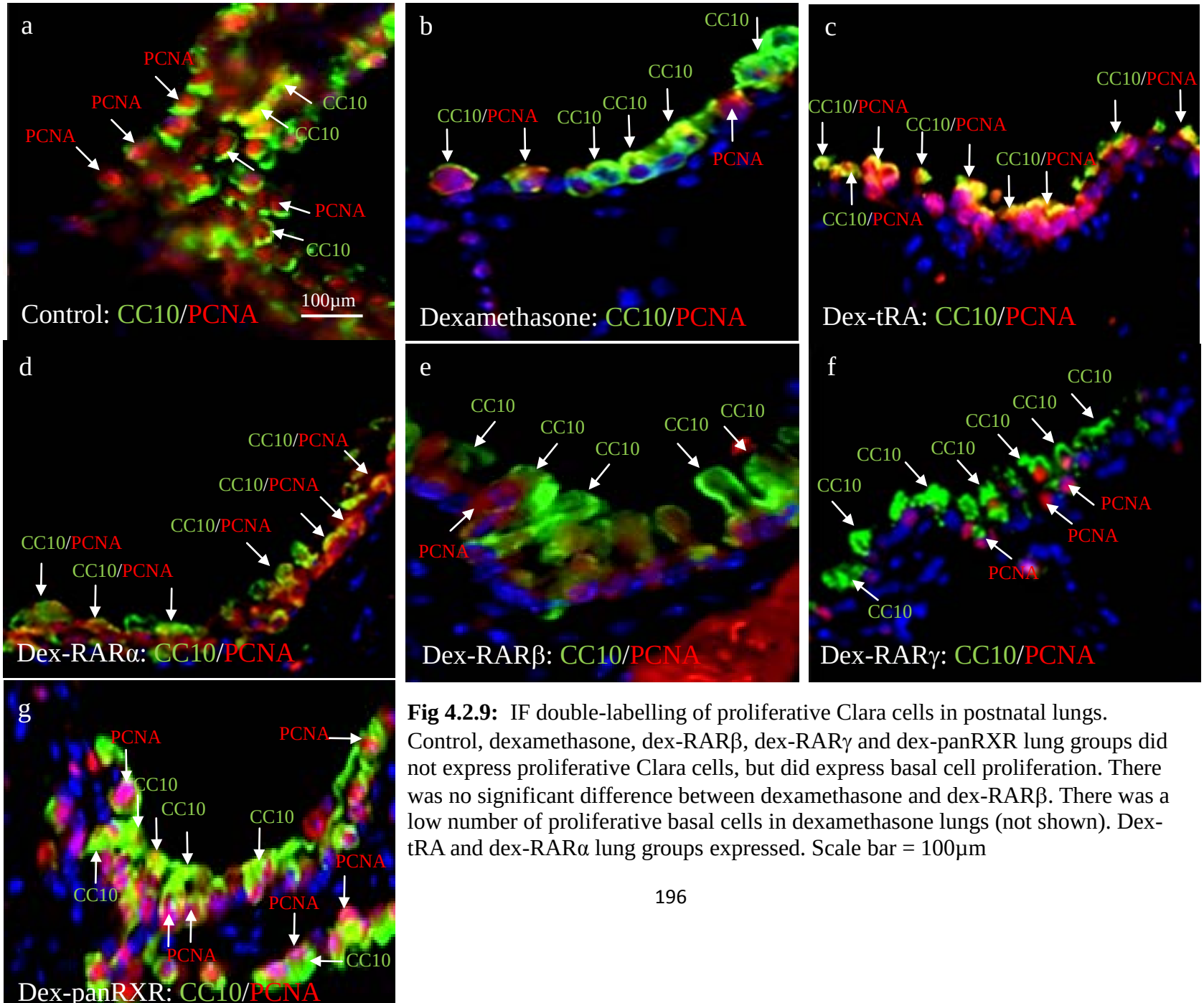

Fig 4.2.9: IF double-labelling of proliferative Clara cells in postnatal lungs. Control, dexamethasone, dex-RARβ, dex-RARγ and dex-panRXR lung groups did not express proliferative Clara cells, but did express basal cell proliferation. There was no significant difference between dexamethasone and dex-RARβ. There was a low number of proliferative basal cells in dexamethasone lungs (not shown). Dex-tRA and dex-RARα lung groups expressed. Scale bar = 100µm

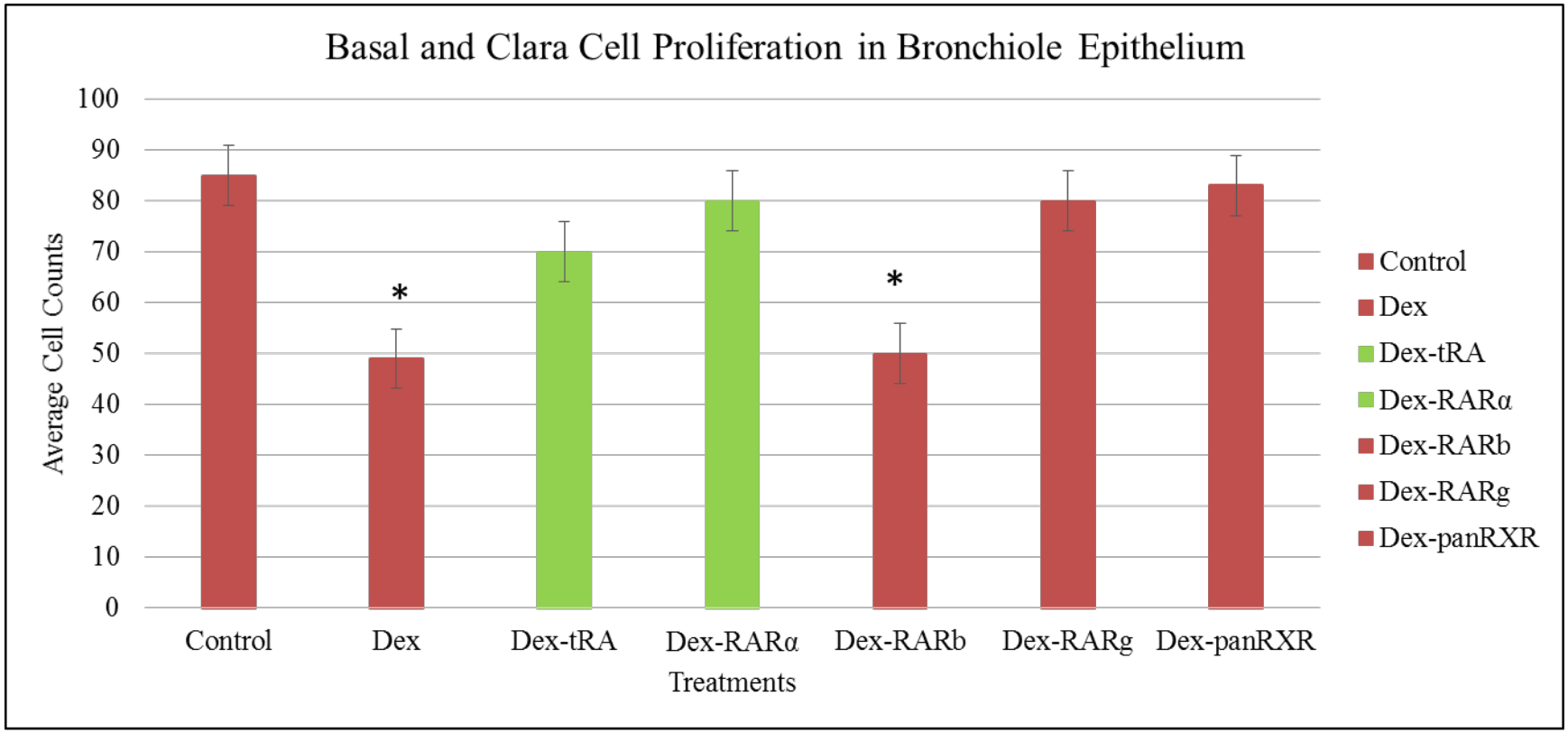

Fig 4.2.10: Basal (red) and Clara Cell (green) proliferation in the Bronchiole Airway Epithelia. Dex-tRA and dex-RARα were the only lung groups that demonstrated Clara cell proliferation. The other retinoid ligand s showed basal cell proliferation. This data suggests that both tRA and RARα promotes bronchiole airway repair/regeneration via Clara cells and that RARβ, RARγ and panRXR promote bronchiole airway repair/regeneration via basal cells.

Table 4.2.4: One-way ANOVA followed by Bonferroni post-correction test for Basal/CC10 proliferation in Bronchiole

Bronchiole	**Control**	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	85	49	70	80	50	80	83
SD	8.7	6.8	20.5	16.4	6.3	6.6	7.6
SEM	3.0	1.0	5.0	4.0	1.0	2.0	3.0
N	10	21	8	15	15	7	8

tRA and RARα were the only two lung groups that demonstrated Clara cell proliferation. All the other lung groups demonstrated basal cell proliferation, but not Clara cells.

During epithelial injury, ATII cells are said to not only proliferate, but to also differentiate into ATI one cells. There is evidence to suggest that irritants such as cigarette smoke can induce alternations of surfactant producing cells (Hohlfield et al. 1997). In the dexamethasone lung, the number of proliferative SP-C positive ATII cells had greatly decreased 62.8 ± 2 (Fig 4.2.11b, Fig 4.2.12, Table 4.2.5). This could possibly be due to ATII cells transdifferntiating into ATI cells. Like chapter 3.2, we did not carry out experiments demonstrating cell differentiation with SP-C positive ATII cells, so we can only assume this to be the case. There was a high level of ATII cells in control lungs (Fig 4.2.11a). This is a process not necessary seen in human lungs. High proliferative performance of ATII cells in control lungs maybe mouse species-related as this expression pattern is not seen in all mouse models.

When examining dex-tRA lung group, morphologically, we observed strong proliferative activity of ATII cells (Fig 4.2.511c). There was strong colocalisation of these two proteins within the alveolar airway epithelia. This showed that ATII cells responded well to tRA, suggesting that tRA is a good repair/regenerative agent in this mouse model. Again, no differentiation markers were used to decipher as to whether these cells were also differentiating into ATI cells nor did we stain for ATI cells to suggest restorative population of ATI cells, thus confirming as to whether we could conclude that there was alveolar epithelia repair. Nevertheless, this result was a good starting point for examining regenerative epithelia in this lung group. The average cell count from this group was 84.2 ± 2.2 confirming high proliferative performance in this group (Fig 4.2.12, Table 4.2.5). Dex-RARα lung group showed a high degree of colocalisation between PCNA and SP-C-positive ATII cells (Fig

4.2.11d). In fact this group showed hyperproliferative activity of ATII cells because virtually all SP-C-ATII cells were positive for PCNA. There was also strong proliferative activity along the alveolar epithelium which were not SP-C/ATII cells.

Dex-RARγ showed a significant number of proliferating ATII cells in the alveolar epithelia (Fig 4.2.11f). Cells were sparse and the number of proliferative cells was almost half the number of the overall average number of ATII cells present in this group. The average cell count for this group was 78.5 ± 9 (Fig 4.2.12). This was a significantly high difference of proliferative promotion between lung groups. It also gave a statistical p value of $p = <0.01$. The performance of this group matched the performance of dex-tRA lungs, and p value between these two groups was >0.05, making this difference non-significant signifying identical functioning of the groups.

Dex-panRXR lungs showed a number of proliferating ATII cells. The majority of these cells showed proliferation occurring around capillary epithelia (Fig 4.2.11g). This group of lungs showed a clustered population of proliferative ATII cells, but due to the overall morphology of the lungs the actual number of proliferative cells present was not as much as expected. However, this group proved to contain more proliferative ATII cells than in dexamethasone lung groups (Fig 4.2.11b) and dex-RARβ. The average cell count for this group was as follows; 47.4 ± 8.1in comparison with dexamethasone lung group; 62.8 ± 2 (Fig 4.2.12, Table 4.2.5). From this statistical analysis, it showed that this retinoid had made a concerted effort to reverse the effects of dexamethasone damage to the epithelium.

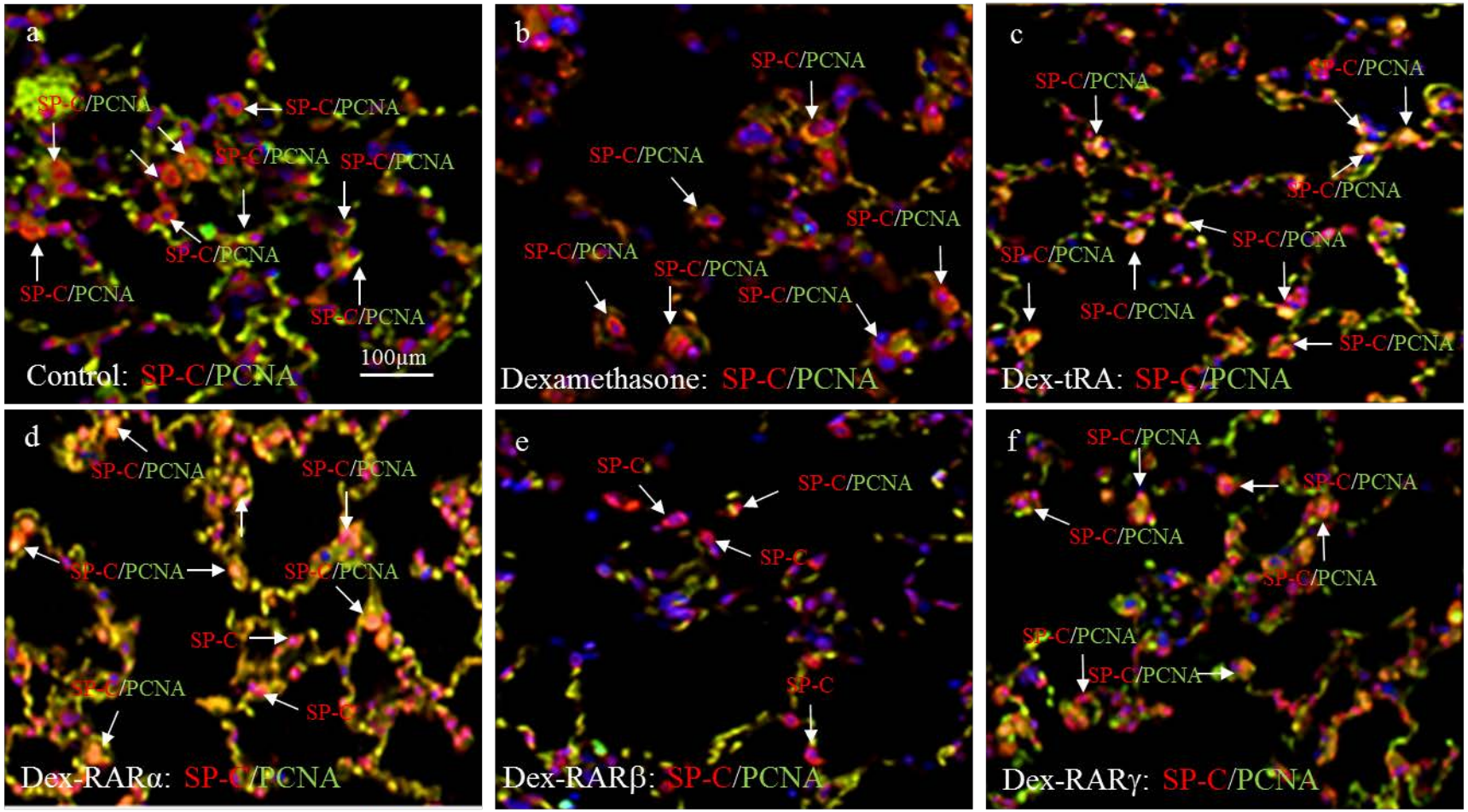

Fig 4.2.11: IF double-labelling of proliferative SP-C/ATII cells in control, dexamethasone and retinoid ligand lung groups. There was strong cell turnover seen in control lungs (a). This was equally seen in dexamethasone lung groups (b), but in lower numbers suggesting proliferative response to injury and perhaps cell differentiation (not seen). Dex-tRA showed a strong response to ATII cell proliferation, dex-RARα also showed a strong response to alveolar epithelial repair/regeneration. There was a very small number of ATII cell proliferation in dex-RARβ (e), but an increase in ATII proliferation in dex-RARγ and dex-panRXR (f and g). Scale bar = 100µm

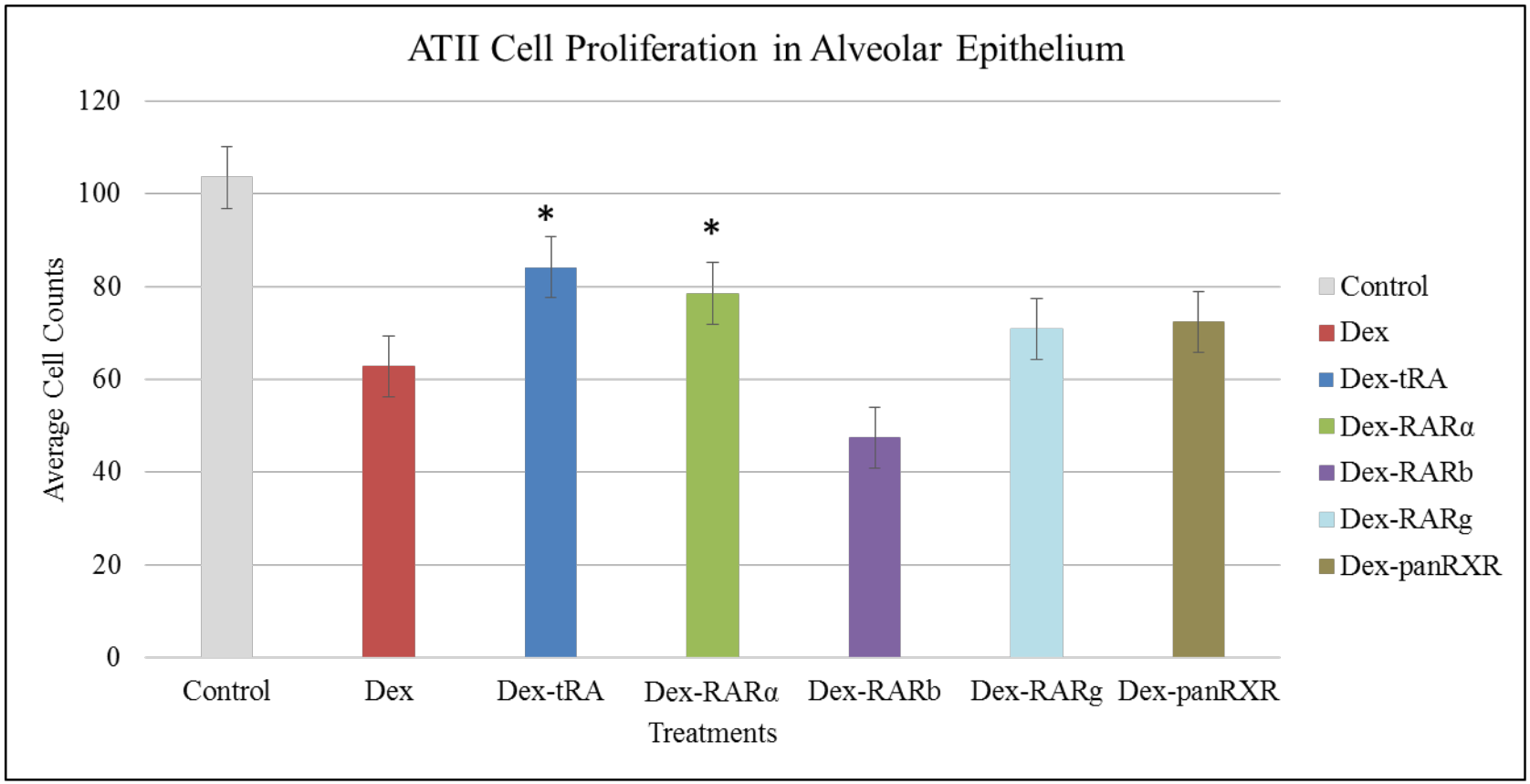

Fig 4.2.12: ATII cell proliferation in the alveolar airway epithelium. Once again, tRA and RARα demonstrated highest upregulation of ATII proliferation in the alveolar epithelium in response to dexamethasone airway damage to the epithelium. All treated lung groups to some degree showed ATII proliferation, but tRA and RARα were shown to be the most efficient RA derivatives.

Table 4.2.5: One-way ANOVA followed by Bonferroni post-correction test for ATII proliferation in Alveolar Epithelium

Alveolar	**Control**	**Dex**	**tRA**	**RARα**	**RARβ**	**RARγ**	**Pan-RXR**
Mean	103.5	62.8	84.2	78.5	47.4	70.8	72.4
SD	6.2	3.4	5.2	6.4	6.3	7.1	6.7
SEM	3.0	1.0	5.0	4.0	1.0	2.0	3.0
N	10	21	8	15	15	7	8

tRA and RARα was shown to the best promoters of proliferative activity in both alveolar and bronchiole (Table 4.2.4) and therefore good promoters of repair/regeneration in the dexamethasone postnatal mouse model.

To further confirm expression changes of Clara and ATII progenitor cells along with their transcription and proliferative activity in dexamethasone and retinoid groups, we measured the protein levels. Treatment of dexamethasone was shown to slightly increase in CC10 levels, but a decrease in the expression levels of SP-C and TTF-1, strongly suggesting that in postnatal mouse lungs, surfactant is still regulated by TTF-1 transcription activity, but Clara cells are dependent of this transcription factor (Fig 4.2.13, lane 2). There was also an increase in proliferation in comparison to control groups (Lane 1). When comparing CC10 and SP-C expression levels in regenerative groups (Lanes 3-7), CC10 expression levels were much lower in all five groups compared to dexamethasone and control groups. However, when examining SP-C expression, dex-tRA and dex-RARα lungs showed upregulation in comparison with dexamethasone groups, followed closely by dex-panRXR. Dex-RARβ was shown to have no improvement on the promotion of SP-C after dexamethasone treatment. Again, expression of TTF-1 mirrored similar results to SP-C expression levels where increase expression levels were observed in dex-tRA and dex-RARα and to a lesser extent, dex-panRXR lungs. All seven groups showed proliferative activity, dexamethasone and dex-RARβ lung groups showed the least amount of activity compared with the other lung groups.

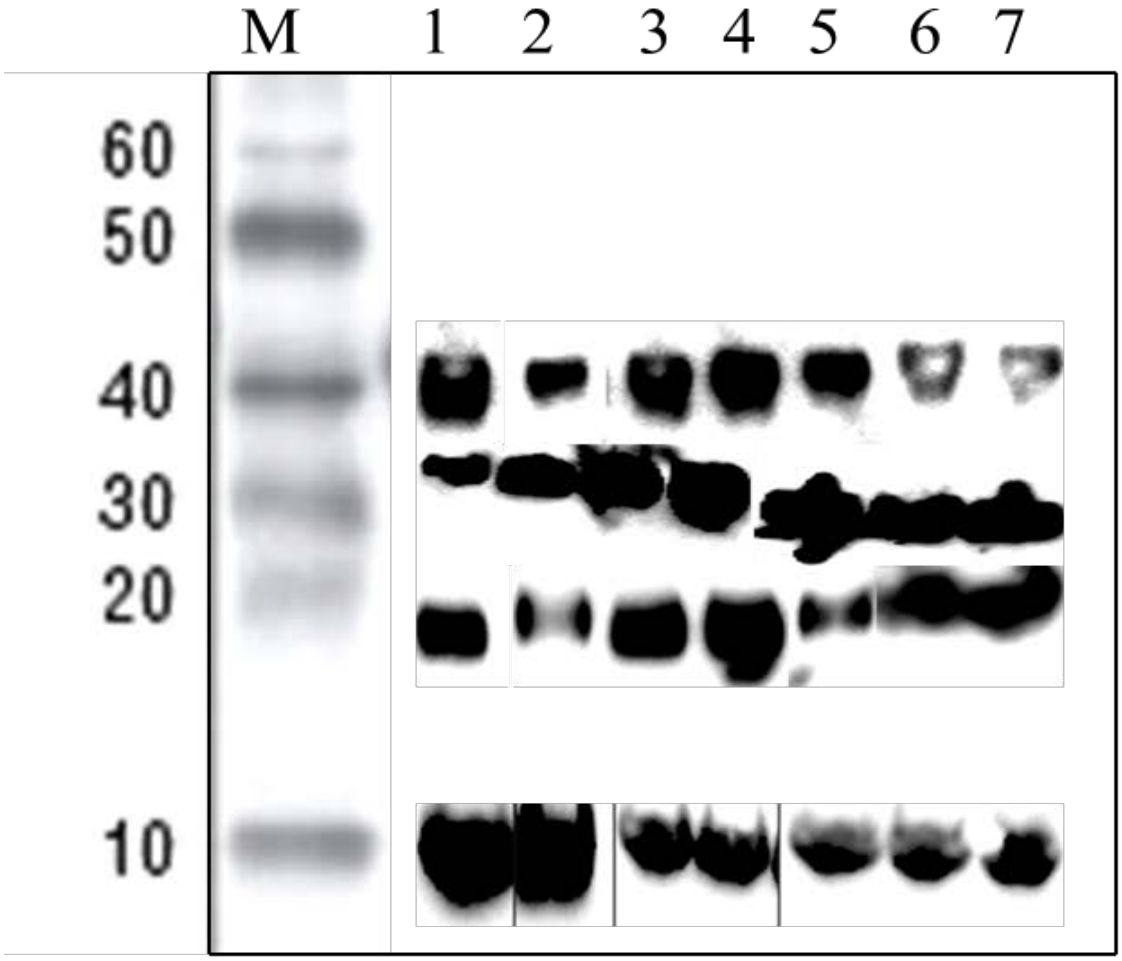

Fig 4.2.13: Western blot analysis of mouse anti-TTF-1 (29kDa), rabbit anti PCNA (29kDa), rabbit anti-SP-C (21kDa) and goat anti-CC10 (10kDa) in postnatal dexamethasone mouse model. Immunoreactive bands were detected with HRP goat anti mouse, goat anti rabbit and rabbit anti goat antibody. Molecular weight standards are indicated to the left lane (Lane M). Lane 1: Control, Lane 2: Dexamethasone and Lanes 3-7 regenerative retinoid ligands. tRA and RARα lung homogenates show promotion of epithelial cell renewal through upregulation of proliferation and transcriptional activity.

4.2.5: DISCUSSION. Pulmonary lung diseases are difficult to understand because there are such numerous components that each has its role in the clinical features that is poorly understood at a molecular level. The cause is often unknown and diagnosis through morphology may represent different disease processes. Both bronchiole and alveolar epithelia are early sites of damage after pulmonary insult. Repair of the alveolar epithelium is thought to occur by the proliferative activity of ATII cells with subsequent differentiation into ATII cells, thus restoring the normal air/liquid interfacial barrier. There is a need, particularly in lung diseases such as COPD/emphysema, to find effective and long lasting therapeutic treatments as there is currently none available. One such candidate for the task is RA and its natural and synthetic analogue; the retinoid receptor agonist. Vitamin A is essential for normal development and maintenance of epithelial tissue including the respiratory system. Retinoids mediate a variety of essential cellular processes including embryogenesis and also influences cell growth and differentiation of adult cells (Swift, Hays et al. 2008). These processes are mediated by retinoid receptors, members of the steroid hormone nuclear receptor superfamily, the retinoic acid receptors, RARs and the retinoid X receptors, RXRs.

As the airway epithelium is critical to the normal functioning of the lung, attempts to study this tissue layer have been carried out for decades. A number of animal and human in vivo and in vitro models have been elevated which mimic the airway epithelium in the postnatal body and which might contribute to an in-depth level of understanding of injury repair mechanism. Relevant models of the airway epithelium could also enable drug metabolism studies, the dissection of pathways important in

control of permeability and the study of a wide range of cellular processes at a molecular level.

Pulmonary surfactant and Clara cell secretory protein (CCSP) are synthesised and secreted primarily by ATII and Clara cells in alveoli and bronchiole epithelium in lungs. Deficiency or disruption of these secretory proteins causes respiratory distress. Surfactant facilitates the spreading and enhances the stability of phospholipids in the alveoli and plays an important role in host defence; the role of CCSP is mainly defence in the form of anti-inflammatory and immunomodulatory functions. Transcriptional regulation of surfactant and CCSP proteins by hormones and tissue-specific transcription factors is the key step for elucidation of surfactant homeostasis in lung development and postnatal respiratory adaptation. We examined pattern of expression of Clara and ATII cells in our dexamethasone-induced injured postnatal lungs with the use of retinoid ligands for the promotion of alveoli and bronchiole epithelial repair/regeneration.

The pattern of expression of SP-C positive ATII cells was significantly altered and exhibited a patchy appearance in these damaged lungs. Effective proliferation and differentiation of ATII cells is thought to be critical for both repair of damaged alveolar epithelium and the prevention of intra-alveolar fibrous tissue deposition. The loss of SP-C protein expression in some areas of this lung group may represent areas where attempted ATII cells repair was not sufficient in restoring the normal alveolar space. It is also possible that these areas contained less differentiated ATII cells that lack SP-C protein. The airway epithelium in this model be have been subjected to a higher dose of dexamethasone due to the subcutaneous route of

administration, thus enhancing the toxic effect. Therefore the examination of the airway epithelium is an important step in determining the applicability of this model of lung injury in human respiratory situations.

Examination of CC10 in dexamethasone lungs revealed severe damage to the bronchiole airway epithelia and may have exacerbated an inflammatory response which is thought to set the stage for further damage, i.e. the development of fibrosis.

We examined the bronchiole epithelium for changes in the distribution and altered morphology of Clara cells. There was a significant increase in the number of Clara cells present in this group of lungs, whereas in studies where for example, bleomycin treatment has been employed to create injury, the results have shown a dramatic loss in Clara cell production although bleomycin showed epithelial cell damage, a feature also seen in the dexamethasone model (Daly et al. 1996). This finding could be related to the nature of dexamethasone, as in normal circumstances, it is used as an anti-inflammatory drug to treat inflammatory and autoimmune conditions. It has been suggested that dexamethasone does not suppress Clara cell production, but does severely alter the morphology and therefore the overall functional role of this cell, but how, if true requires further investigations.

There was additional loss of Clara cells expression in all three lung groups. It may be that in the case of the retinoid ligands maybe sensitive or respond differently to toxic agents within the Clara cell population, or that subpopulations of Clara cells are produced in response to retinoid activity. The pronounced loss of CC10 protein

in the bronchiole airway epithelia in this model may be due to the route of administration of these retinoids and differential Clara cell sensitivity.

In tRA and RARα lung groups, there were significantly higher numbers of these cells with high proliferative activity, which mimicked CC10 protein expression seen in the bronchiole airway epithelium. This highly productive activity of both progenitor cell populations in these retinoids is suggestive that both tRA and RARα are strongly involved in alveolar and bronchiole airway epithelial remodelling. ATII proliferation was seen in the other three retinoid agonists RARβ, RARγ and panRXR, but the level of SP-C protein expression did not match levels seen on tRA or RARα.

tRA and RARα was shown to be important for the stimulation of SP-C protein expression at transcriptional level. RAR signalling pathway is well known to be critical in epithelial cell differentiation and proliferation on many tissue, but little is known about the pathway interacts with and is determined by tissue-specific factors in the respiratory system. We demonstrated that tRA and RARα stimulates both CC10 (Clara cells) and SP-C (ATII cells) through TTF-1 (Nkx2.1) transcriptional activity. To determine whether other RAR ligands directly influenced interaction of Clara and ATII cells with Nkx2.1, we examined RARβ, RARγ and panRXR. These ligands gave mixed results. Expression of Clara and ATII cells in RAR, RARγ and panRXR lung groups appeared to be independent of proliferation and transcriptional activity. It is true that these ligands produced less Clara and ATII cell stimuli, but the level of Nkx2.1 expression was even less. Proliferative activity was unusually high in RARβ. RARβ is known to inhibit proliferation especially in tumorigenesis

i.e. a growth inhibitor, however, this group did fail to show any proliferative relationship between Clara and ATII cells. RARα was the only ligand that showed both sets of progenitor cell populations to be dependent upon Nkx2.1 transcriptional activity postnatally.

Bronchiole and alveolar epithelial repair was shown to be taking place in tRA and RARα lung groups where there was co-localisation of PCNA and CC10/SP-C - positive Clara and ATII cells. Proliferative cells in the bronchioles of dexamethasone lungs was restricted to basal cells. The fact that basal cell proliferation was present in dexamethasone lungs may suggest that basal cells have a role in bronchiole epithelial repair in this animal injured model. Clara cell structure in dexamethasone lungs were altered in morphology. There is a possibility that these cells detected were a subset variant of what we know are Clara cells. Interestingly, we also observed the same fate of Clara/basal cell population in RARβ, RARγ nor pan-RXR treated lung groups. Morphology, Clara cells in these groups also showed alterations. This observation could merely suggest that these ligands have no direct response to dexamethasone damage inflicted on the bronchiole epithelia in terms of progenitor cell activation, but are more likely to be involved in basal cell activation in the repair/regeneration of bronchiole epithelia repair of this model.

Data generated from IF labelled SP-C-positive ATII cells in dexamethasone treated lung group suggest that ATII cells function as progenitor cells in the alveoli and proliferate and differentiate into ATI cells. Single fluorescent labelling showed some areas to demonstrate squamous cells (ATI) along the epithelium of alveoli alongside cuboidal cells (ATII) embedded in the alveoli epithelium. This observation may

explain the reduced number of ATII cells detected via IF and immunoblot in these lungs. Similarly with CC10-positive Clara cell results, both tRA and RARα showed higher levels of ATII population and high proliferative activity. This highly productive activity of proliferative progenitor cells in tRA and RARα is suggestive that these two ligands are strongly involved in airway epithelial remodelling. ATII proliferative activity in the other three ligands were also observed, but the level of SP-C protein was much reduced in comparison with tRA and RARα. This implies that these ligands, RARβ, γ and panRXR give rise to only small clones of daughter cells and that there is a low rate of differentiation into ATI cells. Interestingly, while ATII cells do give rise to ATI during retinoid-induced repair, the rate of differentiation of RARβ and RARγ appeared significantly lower than during dexamethasone-induced lung injury. This puts forward the idea that dexamethasone injury promotes the production of local signals that enhance the conversion of ATII cells to ATI cells.

RAR stimulation on target proteins in the process of repair/regeneration depends on multiple proteins factors interacting with various functional domains. In addition to tRA and RARα ligand interacting with Nkx2.1, tRA and RARα strongly interacts with proliferation factors including PCNA. tRA and RARα interact with other transcriptional and proliferation factors, but these were not explored in this study with dexamethasone mouse model, but is worthwhile examining.

4.2.6: SUMMARY. Chronic lung injury leads to permanent alternations to both cellular and extracellular components that lead to a permanent decline in lung

function. In asthma and COPD, it has been shown that airway epithelial damage contributes to airway remodelling possibly due to altered regulation of the repair process (Jeffery 2001, Holgate, Holloway et al. 2004). Regeneration/repair involves two processes; cell migration and cell proliferation. There are numerous studies where SP-C protein has been seen expressed in alveolar ducts suggesting that this cell population to represent a progenitor cell population for both Clara and ATII cells in the developing and in damaged lungs. In the case of the dexamethasone mouse model we did not witness either cell populations migrating into each other's respective epithelium domain. Nevertheless, these airway epithelial cells are unique in their expression of SP-C protein and CC10 proteins and may represent progenitor cell types as important in epithelial regeneration.

CHAPTER 4.3

EXPRESSION OF MUCINS IN RETINOID TREATED POSTNATAL MOUSE LUNGS

OBJECTIVE: Airway mucus protects and maintains the health of the respiratory system. Its production is orchestrated by the environment, therefore individual variations in mucus composition, quantity and rheology is likely to confer differences in disease susceptibility and response and may also result in environmental specific suitability. Glycoproteins, known as mucins are considered to be the major components of mucus.

In this study we examined changes in the distribution of secretory glycoproteins; acidic and neutral secreted from goblet cells in dexamethasone and retinoid-treated groups and compared with control and retinoic acid (tRA) groups. We also examined the major airway mucins normally found in healthy lungs (MUC5AC and MUC5B) as well as mucins associated with injury to the airways (MUC2 and MUC7). A predominate population of acidic glycoprotein was observed in dexamethasone and dex-panRXR treated lungs. Other retinoid ligands dex-RARα, dex-RARβ and dex-RARγ showed a mixed population of acidic and neutral glycoproteins of varying degrees. Further examination of mucin proteins showed an increase in MUC2, MUC5B and MUC7 protein expression in dexamethasone treated lungs. There were mixed results with retinoid treated lungs. MUC2, MUC5B and MUC7 showed an improvement in expression in all retinoid lungs in comparison to dexamethasone treated lungs. MUC5AC was inhibited by dex-tRA and dex-RARβ lungs, but displayed similar reduced levels with Dex-RARα and dex-panRXR lungs that were almost similar to control lungs. Dex-RARγ showed upregulation of MUC5AC.

Variability in results was sufficient to demonstrate that mucin glycoproteins are a major constituent of airway insult associated with mucociliary clearance, changes in the composition of mucin glycoproteins and that a balance in population is vital for normal lung function.

4.3.1: INTRODUCTION. Mucus hypersecretion is a feature pathologically linked to chronic respiratory diseases such as asthma, cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) (Vestbo 2002, Turner and Jones 2009). The pathophysiology of this feature is defined as airway obstruction, airway flow limitation, ventilation-perfusion mismatch and impairment of gas-exchange (Rogers 2007). In the large airways (tracheobronchial tree), mucus is produced in goblet cells and submucosal glands whereas in the small airways (bronchioles) the only source of mucus secretion is in the goblet cells (Rogers 2007, Turner and Jones 2009).

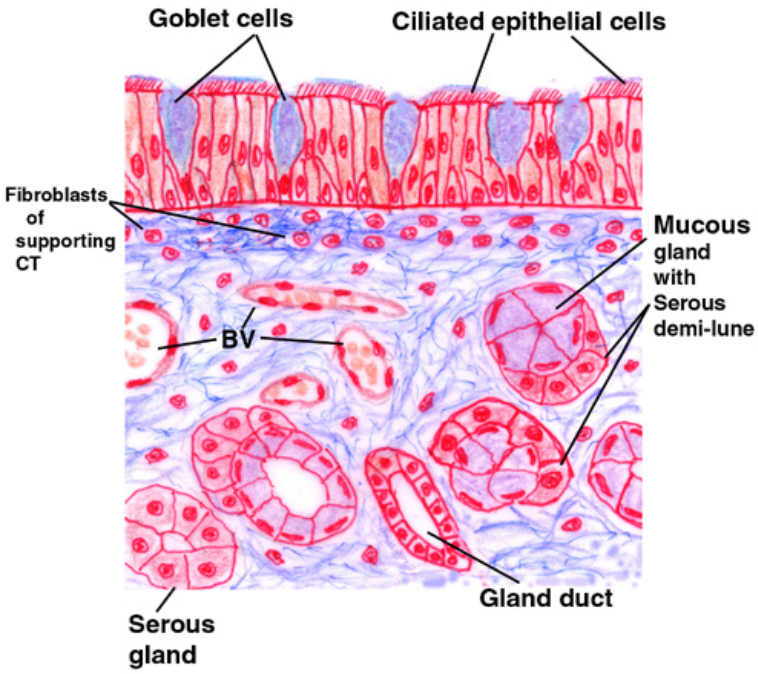

Fig 4.3.1: Typical respiratory epithelium. Pseudostratified columnar epithelium with goblet cells scattered throughout. *Caceci T. 2005. Veterinary histology: Respiratory system study*

Airway mucus is composed of two forms, a secretory liquid (periciliary) form that allows the cilia to beat and a gel-form on the surface of the cilia whose thickness varies between 2 and 20μm (Nicod 1999). The flow of the gel forming mucus is referred to as the mucociliary transport (Nicod 1999). The physiological properties of mucus are provided mainly by mucins, which are mucopolysaccharides or glycoproteins and proteoglycans secreted from the surface epithelial cells and glands (Nicod 1999). Phospholipids are also secreted by the epithelial cells and submucosal glands of the airways weakening the adhesion of the mucus, altering its physical properties. The gel forming mucus acts as a barrier to bacteria causing them to adhere to the gel (Nicod 1999). It also protects the airway epithelium from dehydration, chemical particulates and nasally inhaled airway irritants (Leikauf, Borchers et al. 2002).

Normally, only a few mucus-secreting cells are active in healthy individuals. However, in respiratory diseases such as those named above, elevated numbers of goblet cells are coupled with excessive mucus secretion. This increase in goblet secretory cells is often termed as hyperplasia (Jonathan Turner and Carol E Jones. 2009). Following injury by chemical irritants, the initial phase of the restoration of the mucosal epithelium consists of a rapid migration of epithelial cells to the wounded site followed by their spreading to re-establish the integrity of the luminal surface (Leikauf GD et al. 2002). This process has extensively been studied in the intestinal tract, but also occurs in the airways in animal models of airway diseases (Leikauf, Borchers et al. 2002).

Epithelial restoration is mediated in part by a family of small, apically secreted peptides and distinctive disulfide-bonded domain structures of which creates a trefoil configuration that confers resistance to degradation by proteases and extremities of pH. The histochemical evaluation of mucins has revealed a heterogeneous population comprising of neutral and acidic glycoproteins. The use of the simple histological technique, Alcian Blue Periodic Schiff's Reagent (ABPAS) has enable us visualise this heterogeneous population. Studies of airway glycoproteins in lung disease have demonstrated shifts in the proportion of this heterogeneous environment to one mostly consisting of acidic glycoproteins and also a change in goblet cell size.

The two major airway mucin proteins are the gel forming MUC5AC and MUC5B. MUC5AC is normally located in goblet cells, whereas MUC5B is essentially detected in the mucosal cells of the submucosal glands and gland ducts (Copin, Buisine et al. 2001). Therefore it is generally thought that MUC5AC expression is stronger in the trachea and main bronchi where goblet cells are plentiful (Copin, Buisine et al. 2001). The intensity of the expression of MUC5AC decreases in the epithelium of segmental bronchi, but remains strong at the joining proximal ciliated duct and surface epithelium, while MUC5B is expressed in some goblet cells (Copin, Buisine et al. 2001). MUC2 protein is generally weakly expressed on the surface of the airway epithelium, in some goblet cells and within the submucosal glands in mucus cells as well as in some of the serous cells; therefore, MUC2 is not generally thought as being a prominent secretory gel-forming mucin protein in the respiratory airways (Copin, Buisine et al. 2001). Consequently, numerous studies have suggested that MUC2 may have a role in the pathogenesis of inflammatory

airway disorders (Fig 4.3.2), (Hovenberg, Davies et al. 1996, Copin, Buisine et al. 2001). Moreover, transcriptional activation of MUC2 by *Pseudomonas aeruginosa* lipopolysaccharide may play a role in the pathogenesis of CF (Li, Dohrman et al. 1997, Copin, Buisine et al. 2001). Transcriptional activation of MUC2 and its location in pluripotential stem cells or basal cells suggest a role of MUC2 in epithelial renewal in airways (Copin, Buisine et al. 2001).

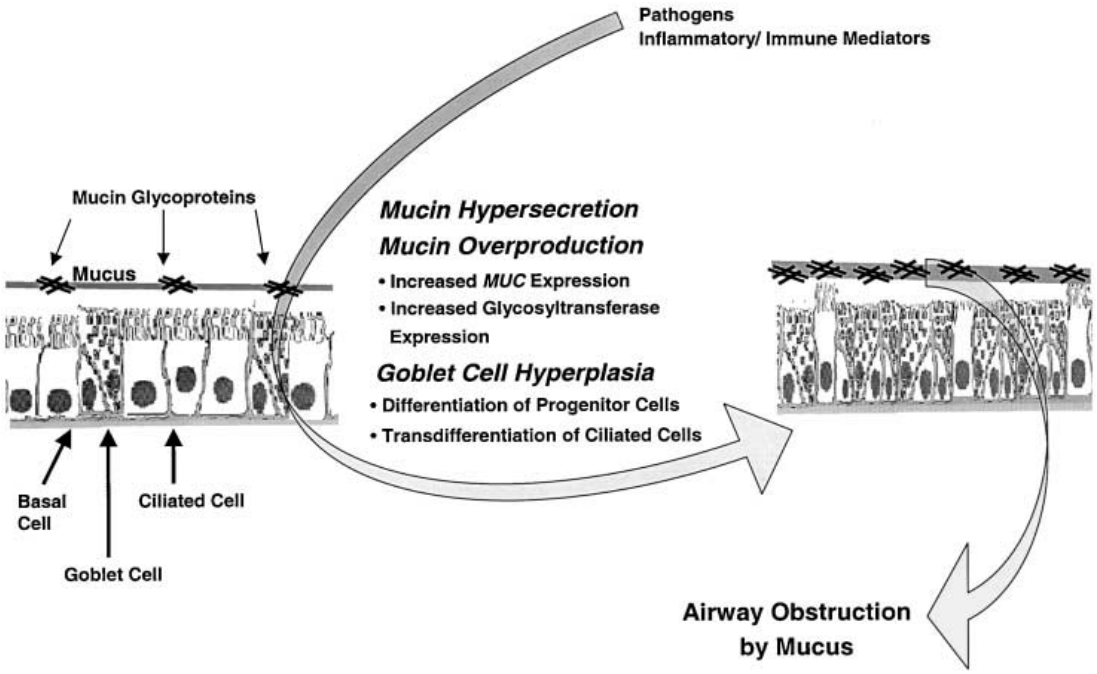

Fig 4.3.2: Processes that impact on mucus obstruction of the airways. A normal airway epithelium (left) exposed to pathogens or environmental agents that activate inflammatory and/or immune mediators may initially respond by mucin hypersecretion from goblet cells and submucosal glandular cells (not depicted). In chronic conditions, hyperplastic airway epithelium (right) results, reflecting division of goblet cells, differentiation of progenitor cells and/or transdifferentiation of airway epithelial cells. *Callaghan-Rose M et al. 2001. Am J Respir. Cell Mol. Biol. 25: 533-537*

MUC5B and MUC7 are defined mutually and exclusively to their cellular compartments within submucosal glands, mucus acini and a subpopulation of serous acini (Copin, Buisine et al. 2001). This differential expression of mucin proteins in submucosal glands might reflect the differences in the differentiation state of these two cell types in response to a variety of airway insults (Copin, Buisine et al. 2001, Rogers 2007).

4.3.2: AIM. The aim of this study was to investigate the importance of mucus in airway function and to establish its effects in injured and lungs undergoing recovery. We showed that in injury, mucus hypersecretion occurred via the lungs 'natural' defence mechanism, but this hypersecretion resulted in causing more damage to the airways than protection. We also observed goblet cell hyperplasia in these lungs. In some of the regenerative lung groups, we a reverse effect of mucus hypersecretion and goblet cell hyperplasia that had occurred in the injured lungs. Previous studies have examined mucus hypersecretion under various conditions and have shown RA to improve mucociliary clearance.

4.3.3: RESULTS: ABPAS staining demonstrated a mix population of acidic and neutral glycoproteins along the bronchi and deep into the bronchiole epithelia of control lungs. Most goblet cells had an oval structure to them. ABPAS staining was easily detected in small amounts (Fig 4.3.3a). This observation reflected the number of actively secreting goblet cell, 5.9 ± 1.0 and 6.1 ± 1.0 (Fig 4.3.4, Table 4.3.1), showing a balanced heterogamous acid to neutral glycoprotein ratio. Goblet cells were effected in two major ways by the administration of dexamethasone. Firstly, goblet cells secreted more acidic glycoproteins and was reflected on the number of AB stained glycoproteins and secondly, goblet cells present in these lungs were mostly columnar in shape (4.3.3b). Compared with control lungs, dexamethasone lungs showed some airway inflammation with an increased amount of luminal mucus. The average of these cell counts in dexamethasone treated lungs were as follows; 27.4 ± 3.4 for acid glycoproteins and 11.5 ± 1.0 for neutral glycoproteins (Fig 4.3.4, Table 4.3.1). This suggest the insult of lung injury caused by dexamethasone not only promotes mucus hypersecretion, but also a change in the airways environment thus effecting mucociliary clearance.

We then examined the response of secretory glycoproteins to dex-tRA. AB-positive acidic glycoproteins showed basal stained cells, but not goblet cells. Goblet cells present in these lungs were shown to mainly secrete neutral glycoproteins. These cells were scattered along the bronchiole epithelium. They were oval in shape, situated on top of AB stained basal cells (4.3.3c). Neutral glycoproteins seemed to play a dominate role in these treated lungs than previously observed in dexamethasone lungs, where it was acidic glycoproteins that was dominate. Despite basal and not goblet cells secreting acidic glycoproteins, we still carried out cell

counts to get an idea of mucus secretion. Cell counts for acidic glycoproteins was 10.5 ± 2.0 and 13.3 ± 11.5 for neutral glycoproteins (Fig 4.3.4c, Table 4.3.1).

In order to get an overall view of the effectiveness of each retinoid ligand after dexamethasone damage, we compared calculated numbers of secretory goblet cells in dex-tRA and dexamethasone lungs. First we examined dex-RARα lungs. Lungs in this group showed a much heterogeneous environment for glycoproteins, although numbers for each protein population was not identical. Numbers of both population were greater than numbers in dex-tRA, suggesting that this particular ligand did not have a direct impact on reducing mucus hypersecretion and therefore mucociliary clearance, but did have a positive effect on a heterogeneity environment for the lung (Fig 4.3.3d). The average cell counts for acidic and neutral glycoprotein secretions in these lungs were 22.3 ± 1.0 (acidic) and 25.5 ± 2.4 (neutral). The significant difference was $p<0.05$ when compared with dex-tRA.

The second retinoid ligand group we examined was dex-RARβ. Secretory goblet cell in this group was much harder to count as there was an abundance of secretory neutral glycoproteins and goblet cells were extremely irregular in shape (Fig 4.3.3e and 4.3.4). Acidic glycoproteins positive for AB were located in basal cells. The bronchiole epithelium of these lungs looked ‘metaplastic’. Goblet cells were oval in shape and quite densely populated. Average cell counts for these lungs were as follows: 15.3 ± 2.3 (acidic) and 28.4 ± 4.3 (neutral). Neutral glycoprotein secretions were almost twice the number of acidic secretions. When comparing these averages with dexamethasone lungs, neutral glycoprotein secretions in dex-RARβ calculated

to be 2.5 times more. The significant difference for this group was $p<0.05$ when compared with dex-tRA (Table 4.3.1).

Next we examined dex-RARγ. This group appeared to have almost equal numbers of acidic and neutral glycoprotein secretions (Fig 4.3.4). Cells that were positive for both populations were oval, but slightly flat in shape and evenly spaced along the respiratory mucosa (4.3.3f). There were areas along the bronchiole epithelia where a mixed population was observed. The average number of secretory glycoproteins in this group was 21.4 ± 2.0 for acidic and 23.0 ± 7.1 for neutral glycoproteins. Even though dex-RARα groups showed similar secretory response, its measurements were greater than dex-RARγ.

The last retinoid ligand group that was examined for glycoprotein secretion was dex-panRXR. The majority of secretory glycoprotein was acidic. Goblet cells appeared relatively tall, but oval in shape (Fig 4.3.3g). Secretory goblet cells were not entirely evenly distributed. There were areas where secretions were highly populated and there were areas where secretory glycoproteins seemed to be situated on the surface of goblet cells. There was very few neutral glycoprotein secretions (data not shown).

The average counts for this group was 30.8 ± 11 for acidic glycoproteins and 7.5 ± 3.4 for neutral glycoproteins. Its acidic secretions were a fraction more than dexamethasone groups with its neutral glycoproteins being slightly less than calculated secretions in the dexamethasone group. When comparing values in dex-

panRXR with values in dex-tRA, they revealed a significant increase of acidic glycoproteins by four times and a decrease of neutral glycoproteins by at least six times (Fig 4.3.4, Table 4.3.1).

The overall measurements of acidic and neutral glycoprotein secretions is summarised in figure 4.3.4, table 4.3.1. The graph (Fig 4.3.4) shows that acidic glycoproteins was greatly stimulate by dexamethasone and dex-panRXR ligands and that neutral glycoproteins was stimulated by dex-RARα and dex-RARβ ligands. The overall measurements of RA ligands indicated that dex-RARγ had the closest stimulatory pattern of reaction to dex-tRA even though the amount of glycoproteins were much higher.

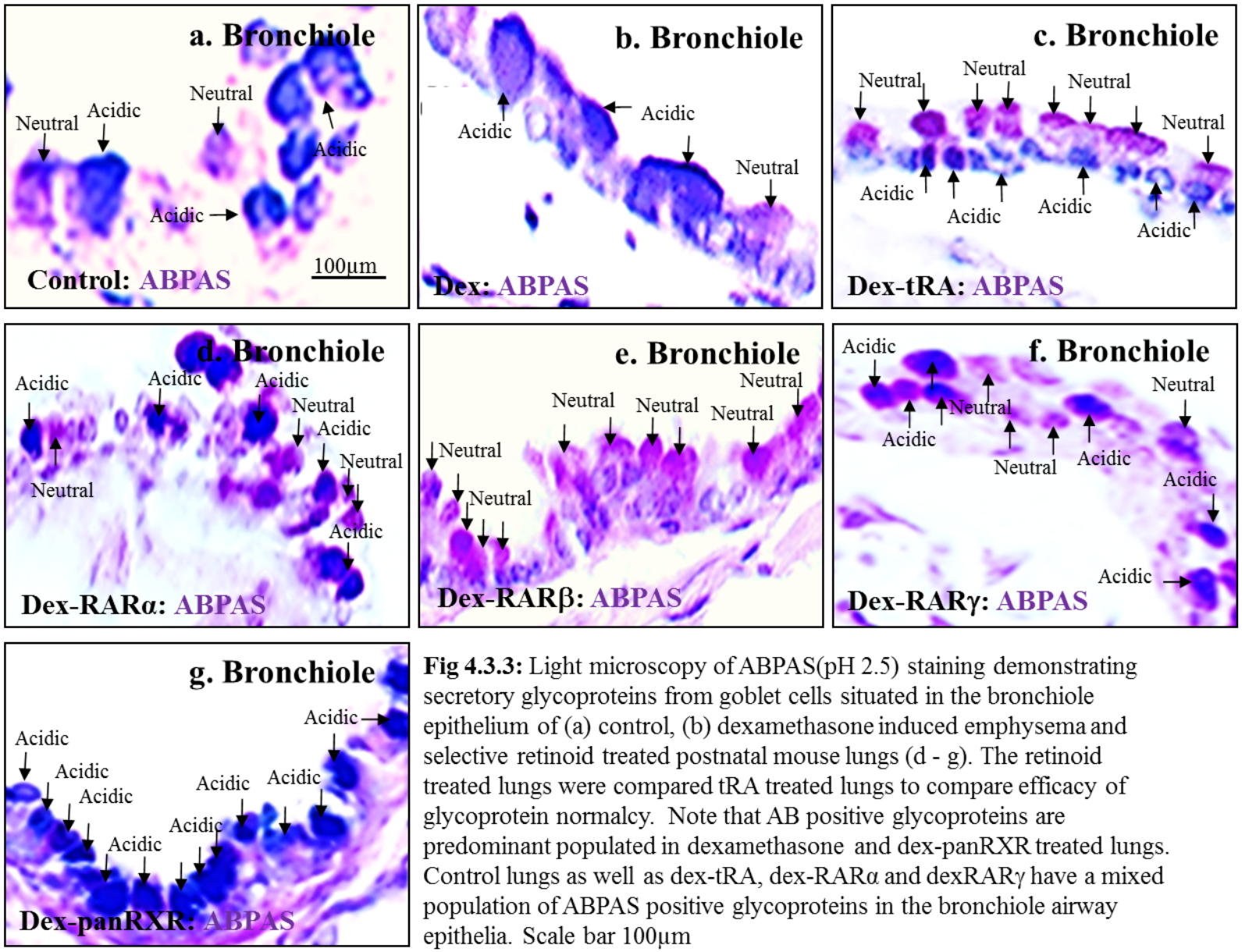

Fig 4.3.3: Light microscopy of ABPAS(pH 2.5) staining demonstrating secretory glycoproteins from goblet cells situated in the bronchiole epithelium of (a) control, (b) dexamethasone induced emphysema and selective retinoid treated postnatal mouse lungs (d - g). The retinoid treated lungs were compared tRA treated lungs to compare efficacy of glycoprotein normalcy. Note that AB positive glycoproteins are predominant populated in dexamethasone and dex-panRXR treated lungs. Control lungs as well as dex-tRA, dex-RARα and dexRARγ have a mixed population of ABPAS positive glycoproteins in the bronchiole airway epithelia. Scale bar 100µm

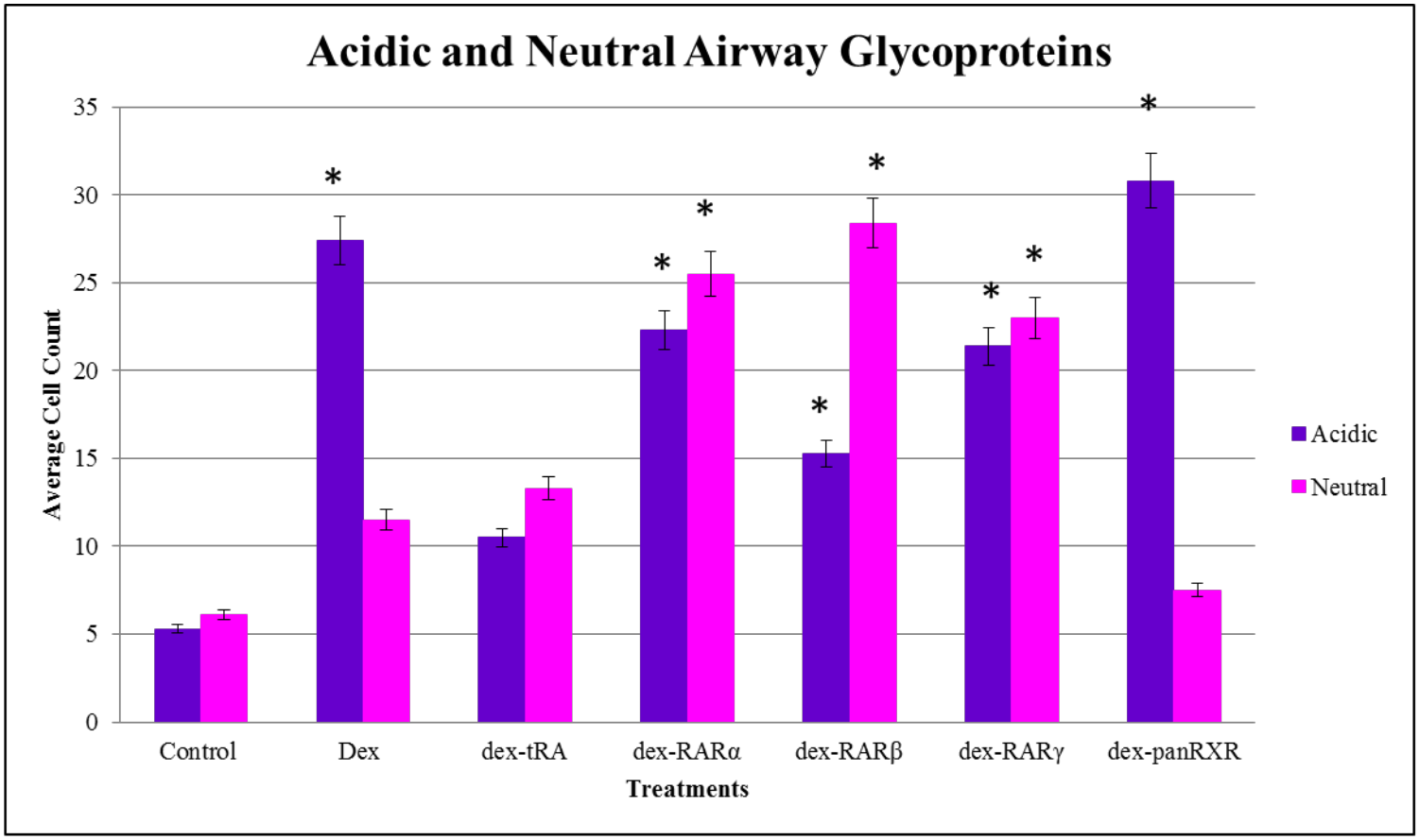

Fig 4.3.4: Acidic and neutral glycoprotein levels in control, dexamethasone and retinoid treated lungs. Dexamethasone and panRXR lungs shows mucus hypersecretion to be dominated by acidic glycoproteins. There are also levels of hypersecretion in some of the retinoid ligand groups, RARα and RARβ that is dominated by neutral glycoproteins. Even though there was an increase in glycoprotein secretion in RARγ, there was not a great difference in comparison with the other ligands.

Table 4.3.1: Acidic and neutral glycoprotein secretions from dexamethasone, and retinoid ligand treated postnatal mouse lungs. Results are expressed as percentage of control, dexamethasone and retinoid ligand lungs, ± standard error of the mean. One way AVONA indicated whether there was a difference in the retinoid ligand groups from tRA lungs. * Indicates a significant difference of acidic and neutral glycoproteins from tRA after a t-test, where $P<0.05$ followed by the Bonferroni correction where $P<0.016$.

Lung Group	N	ACIDIC GLYCOPROTEINS	NEUTRAL GLYCOPROTEINS
Control	10	5.3 ± 1.0	6.1 ± 1.0
Dexamethasone	21	27.4 ± 3.4	11.5 ± 1.0
dex-tRA	8	10.5 ± 2.0	13.3 ± 11.5
dex-RARα	15	22.3* ± 3.0	25.5* ± 2.4
dex-RARβ	15	15.3* ± 2.3	28.4*± 4.3
dex-RARγ	8	21.4* ± 2.0	23.0* ± 7.1
dex-panRXR	7	30.8* ± 11.0	7.5 ± 3.4

We then focused our attention in the investigation of mucin protein expression in samples examined for secretory glycoproteins. We selected four airway mucins, MUC2, MUC5AC, MUC5B and MUC7. As previously mentioned, both MUC5AC and MUC5B are the major muc proteins found in the respiratory system, where MUC5AC is greater than MUC5B under normal conditions. MUC2 and MUC7 are known to be secreted in low volumes in healthy lungs, compared with MUC5AC and MUC5B. In abnormal conditions, MUC2 and MUC7 are known to elevate substantially. Interestingly differentiation and expression of all four proteins have been shown to be RA or retinol-dependent (Gray et al. 2001).

We first examined MUC5B and MUC7 expression levels by using homogenates of our selected retinoid ligand-treated lungs by SDS-PAGE electrophoresis. These samples were loaded onto a gel of equal volume and examined by staining with Coomassie (CBBR20), followed by PAS for the detection of glycoproteins (Fig 4.3.5). MUC5B was the most obvious mucin protein detected by CBB/PAS. As expected with homogenates from control lungs, levels of MUC5B and MUC7 staining intensity was low (Lane 1). Dexamethasone homogenates (Lane 2) showed greater MUC5B expression and a slight increase in MUC7 when compared with control samples. Both dex-tRA and dex-RARα homogenates showed a reduced expression level of MUC5B and MUC7 (Lane 3 and 4). MUC5B staining was relatively strong, but there was no presence of MUC7 in dex-RARβ samples (Lane 5). Both dex-RARγ and dex-panRXR (Lanes 6 and 7) showed an increase in MUC5B when compared with dex-tRA and interestingly, an increase in MUC7.

MUC5B and MUC7 expression were semiquantified using ImageJ followed by one-way ANOVA test. Quantification of MUC5B and MUC7 confirmed expression levels. Calculated bands of intensities are shown in figure 4.3.6, table 4.3.2. Calculated MUC5B band from dexamethasone samples contained the highest protein concentration, 77.5 ± 2 and was significantly different from control samples (45.1 ± 13.9). Dex-tRA and dex-RARα had similar readings, 48.1 ± 12.3 and 49. 4 ± 14.9 respectively and therefore was not significantly different. MUC5B expression levels in dex-RARβ showed a difference compared with dex-tRA (55.1 ± 12.6). Conversely, MUC5B concentration readings from dex-RARγ and dex-panRXR were significantly different from dex-tRA. Both groups were the highest out of the retinoid ligands 69.0 ± 8 and 71.3 ± 9 respectively.

Calculated band intensities for MUC7 were much lower than MUC5B calculated bands and were as follows. Control samples gave an average value of 4.1 ± 1.0. In comparison to control samples, dexamethasone average for MUC7 was 7.5 ± 3.4. Dex-tRA gave an average of 3.3 ±11.5. Average MUC7 band intensity for dex-RARα was 5.5 ± 2.4. Dex-RARβ gave an average less than dex-tRA, 2.4 ± 4.3. Dex-RARγ gave the highest average for MUC7 amongst the retinoid ligands, 9.3 ± 7.1and finally, dex-panRXR MUC7 average was 6.3 ± 2.

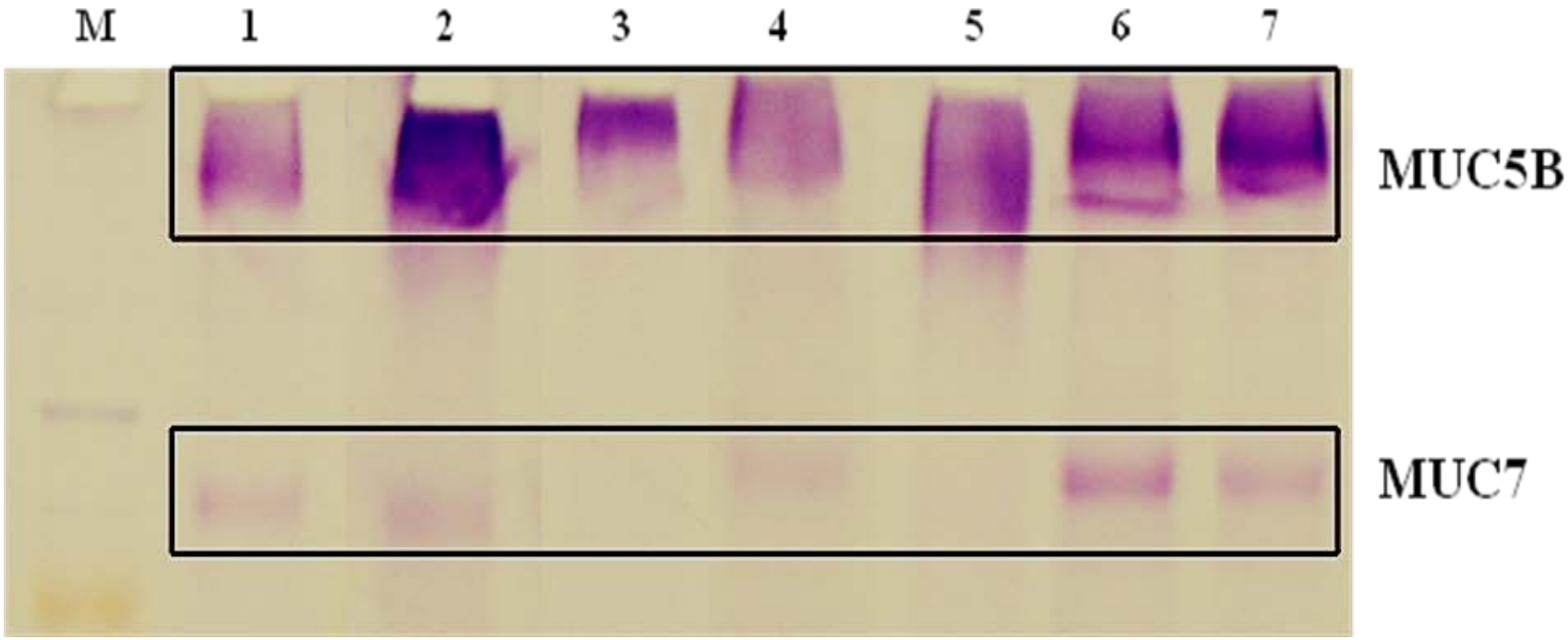

Fig 4.3.5: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrating MUC5B and MUC7 from postnatal mouse lung homogenates stained with Coomassie Brilliant Blue R (CBBR20) and periodic acid Schiff's reagent (PAS) of varying treatments (15µl loaded into each lane). Sample lanes are as follows: Lane 1: Control, Lane 2: Dexamethasone, Lane 3: dex-tRA, Lane 4: dex-RARα, Lane 5: dex-RARβ, Lane 6: dex-RARγ and Lane 7: dex-panRXR. M = Molecular Marker

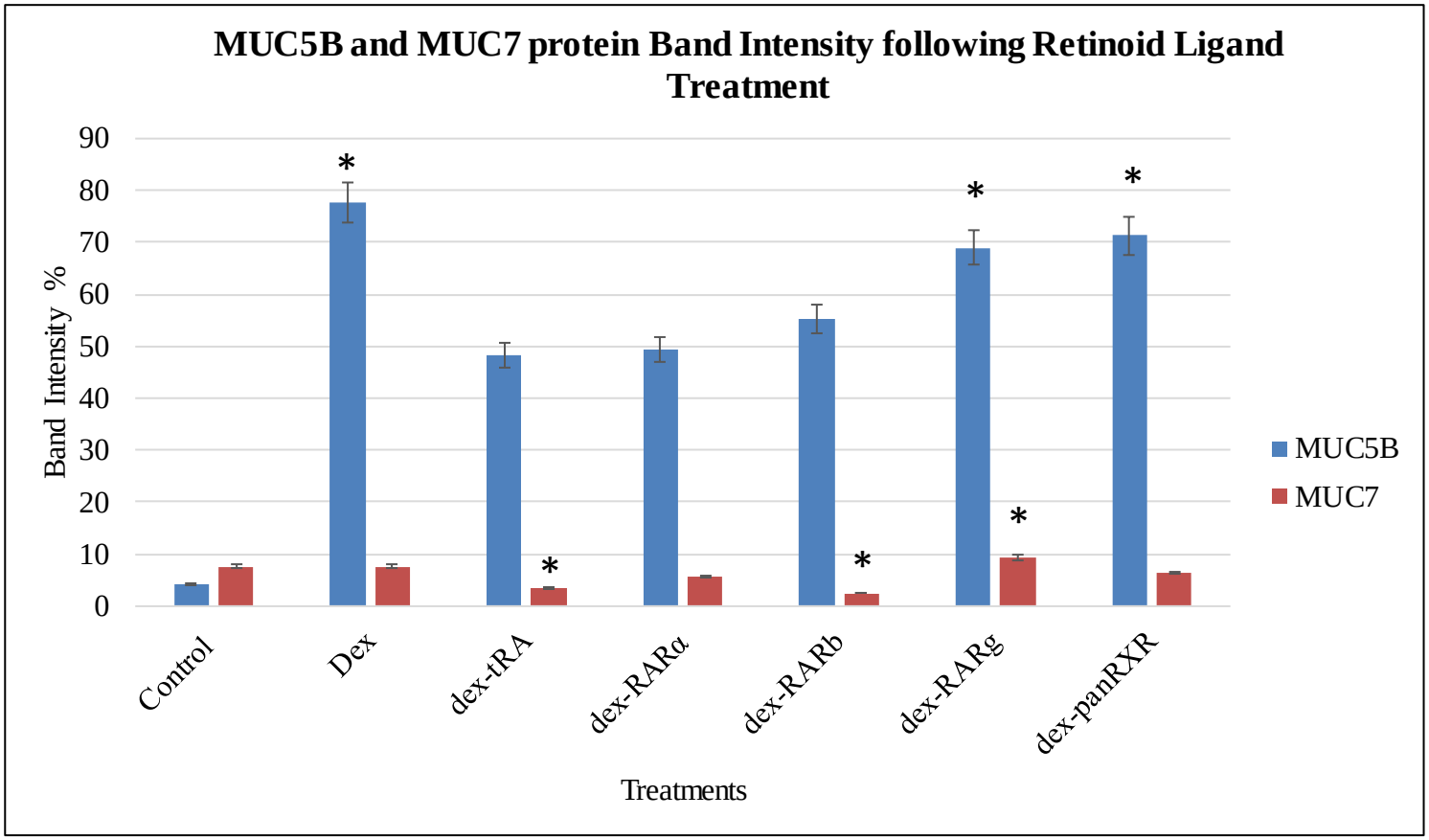

Fig 4.3.6: Graph shows the average band intensity of MUC5B and MUC7 taken from treated lung homogenates. Significant increase of MUC5B in dex-RARγ and dex-panRXR and increase of MUC7 in dex-RARγ following Bonferroni correction. * P<0.01

Table 4.3.2: Band intensity of MUC5B and MUC7 protein following treatment to induce emphysematous-like features and various retinoid ligand treatment to reverse the insult effects of dexamethasone. Table indicates proteins which show a significant increase of both sets of protein. * indicates significance after Bonferroni correction.

Treatment	N	Average MUC5B band Intensity	ANOVA Results	Average MUC7 band Intensity	AVONA Results
Control	10	45.1 ± 13.9	P<0.01	4.1 ± 1.0	Not Significant
Dexamethasone	21	77.5* ± 2.0	P<0.01	7.5 ± 3.4	Not Significant
dex-tRA	8	48.1 ± 12.3	Not Significant	3.3 ± 11.5	P<0.01
dex-RARα	15	49.4 ± 14.9	P<0.01	5.5 ± 2.4	Not Significant
dex-RARβ	15	55.1 ± 12.6	P<0.01	2.4* ± 4.3	P<0.01
dex-RARγ	8	69.0 ± 8.0	P<0.01	9.3 ± 7.1	P<0.01
dex-panRXR	7	71.3 ± 9	P<0.01	6.3 ± 2.0	Not Significant

In addition to Coomassie-PAS staining of MUC5B and MUC7, we examined the expression of MUC2 and MUC5AC in treated lung homogenates via western/immunoblots (Fig 4.3.7, Table 4.3.3). Positive bands were developed using chemiluminescent detection. MUC5B and MUC7 expression levels matched the data obtained from lung homogenate staining with Coomassie/PAS of SDS-PAGE gels. As expected, all four muc proteins showed an increase in expression with dexamethasone homogenate samples (Lane 2). Data generated demonstrated that dexamethasone promotes mucus hypersecretion, in particular MUC2 and MUC5B, where these two muc proteins showed the greatest increase in comparison to control samples. MUC5AC and MUC7 from dexamethasone sample's increase was at a much lesser degree in comparison with control samples. MUC2 and MUC5B responded well to dex-RARα and dex-RARβ treatment (Lanes 4 and 5). MUC7 responded well to all retinoid ligand treated lungs (Lanes 4-7). In control lungs, MUC2 and MUC5AC were mainly seen in epithelial goblet cells, whilst MUC5B and MUC7 were seen in mucus and serous cells of the submucosal gland, respectively. In dexamethasone lungs, goblet cell hyperplasia was observed along with an increase in mucus secretions with all the muc proteins used in this study compared with control lungs (Fig 4.3.5, Lanes 1 and 2).

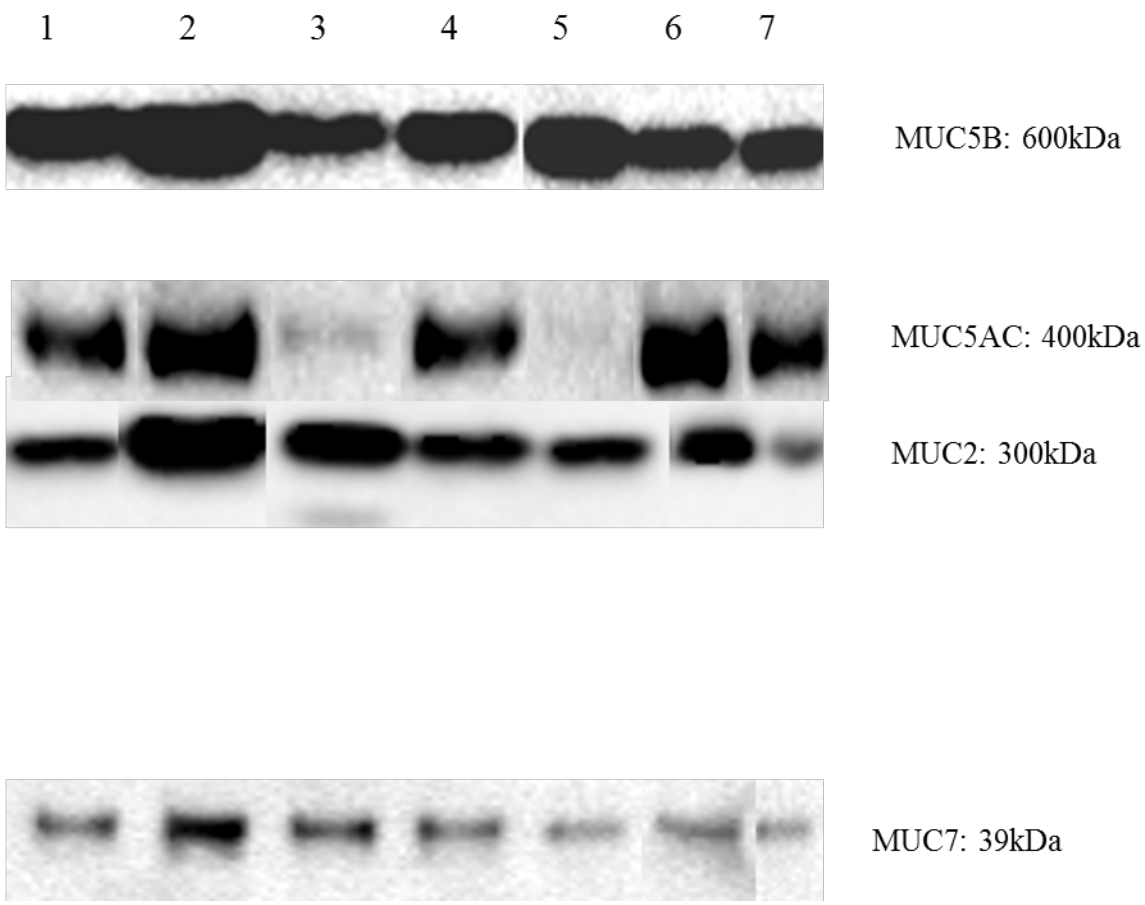

Fig 4.3.7: Immunoblot analysis of mucin proteins in lung homogenates of control, dexamethasone and retinoid treatments. Immunoblots of lung homogenates were probed with antibodies against mucins MUC2, MUC5AC, MUC5B and MUC7. Molecular weights varied from 39 – 600kDa

ANOVA t-test followed by Bonferroni correction confirmed variation of muc protein expression with retinoid ligands (Fig 4.3.8a-d).

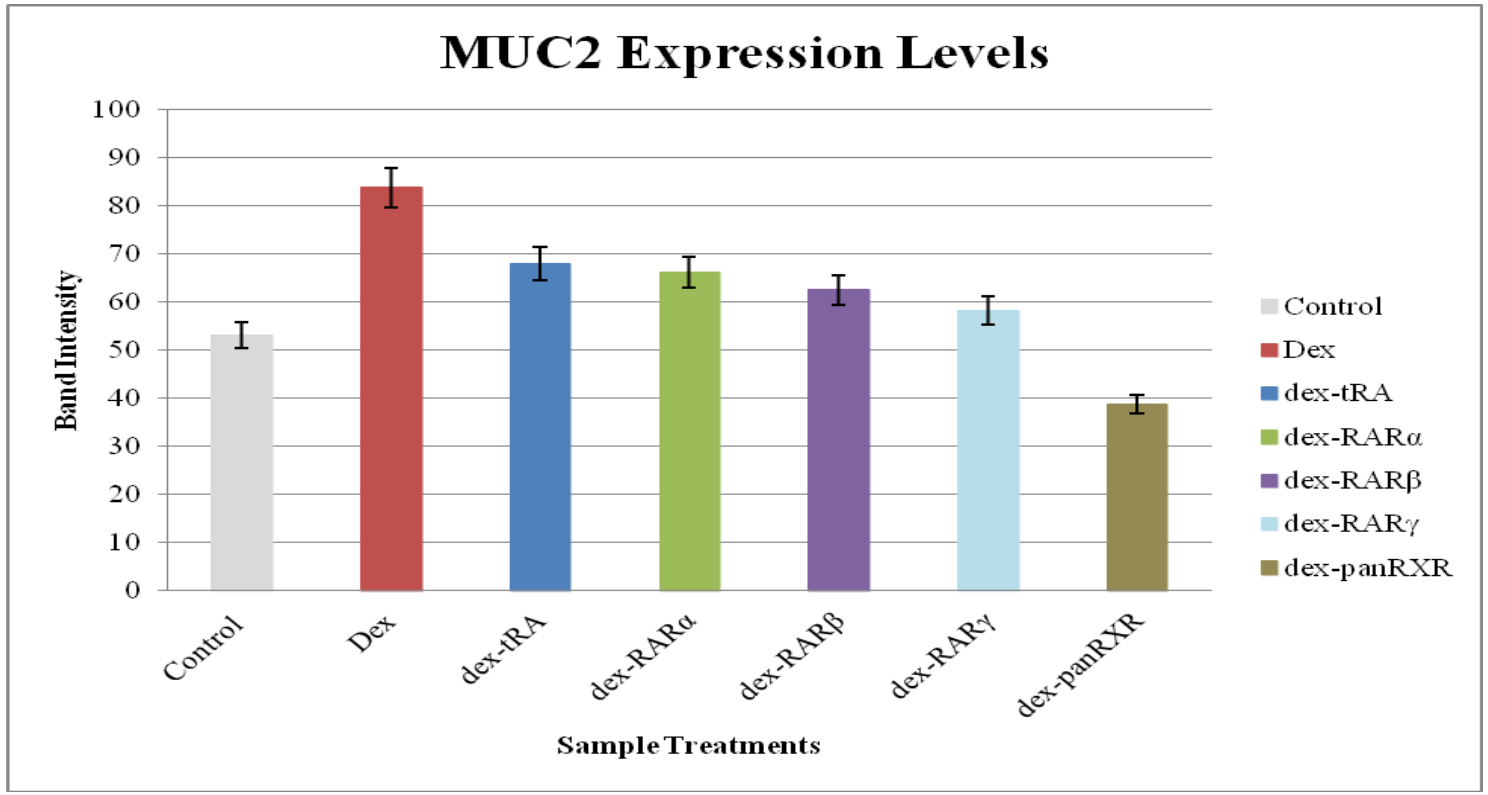

Fig 4.3.8a: Average measurement of MUC2 expression from lung homogenates of varying retinoid ligands. As demonstrated in this graph, MUC2 shows gradual decrease of MUC2 secretion. In terms of comparison with dex-tRA treatment, MUC2 hypersecretion responded well to dex-RARα and dex-RARβ

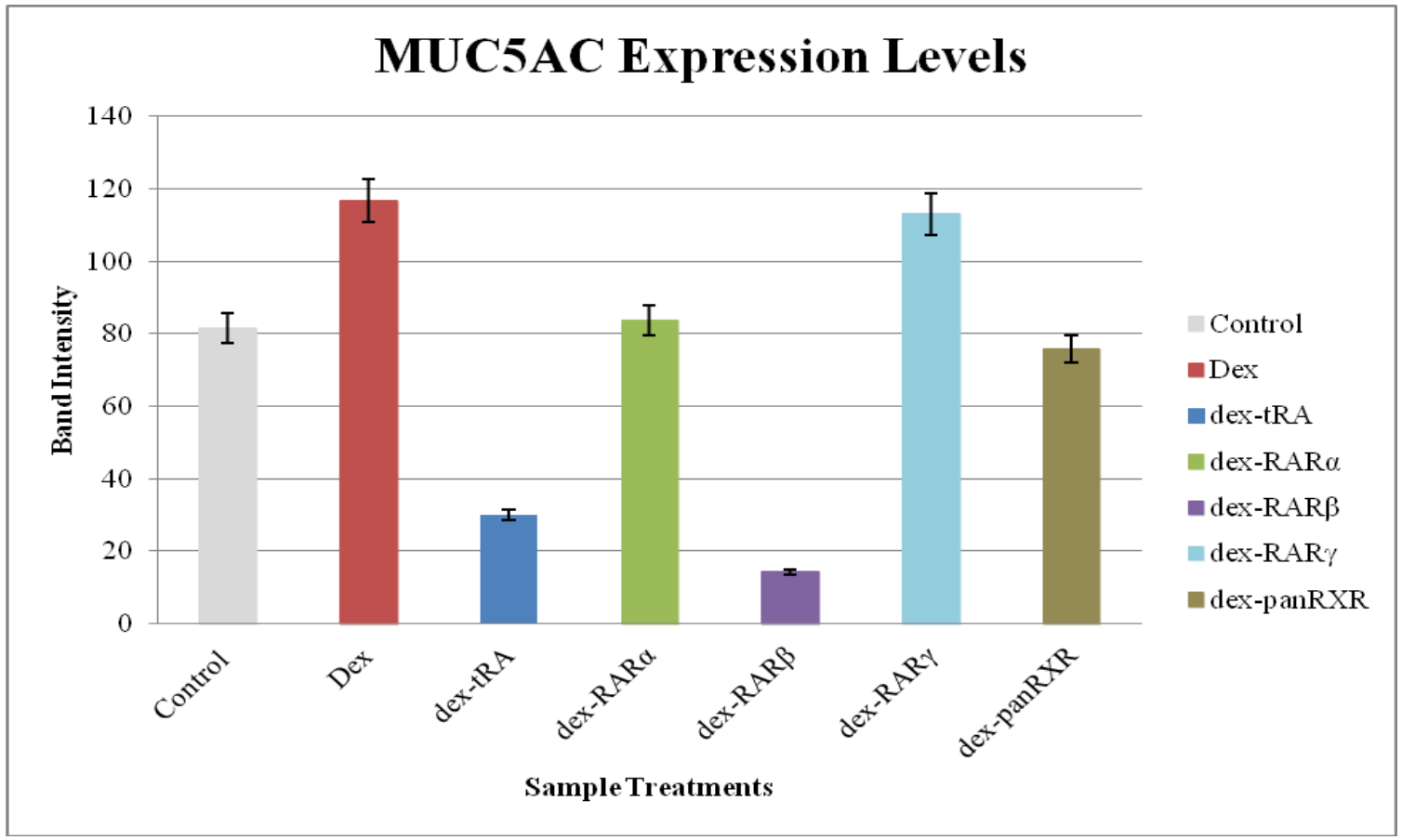

Fig 4.3.8b: Average measurement for MUC5AC expression from lung homogenates from varying retionoid ligands. The graph shows that MUC5AC hypersecretion reponded well to dex-RARα and dex-panRXR in comparison with dex-tRA.

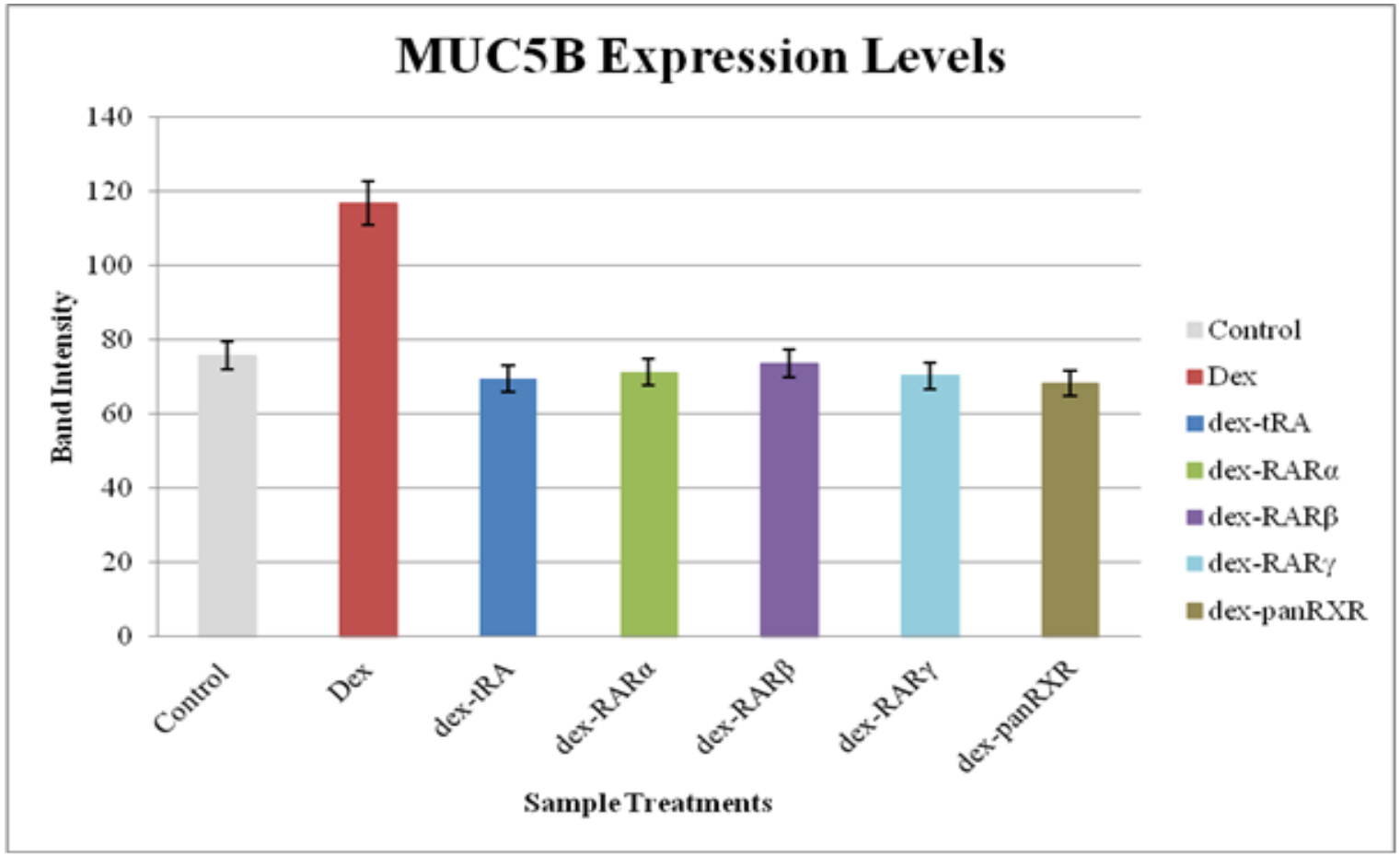

Fig 4.3.8c: Average measurements of MUC5B expression from lung homogenates of varying retinoid ligand treatments. As demonstrated by the graph, dextRA and all of the retinoid ligands have similar expression levels. Dexamethasone was shown to grossly increase MUC5B secretions.

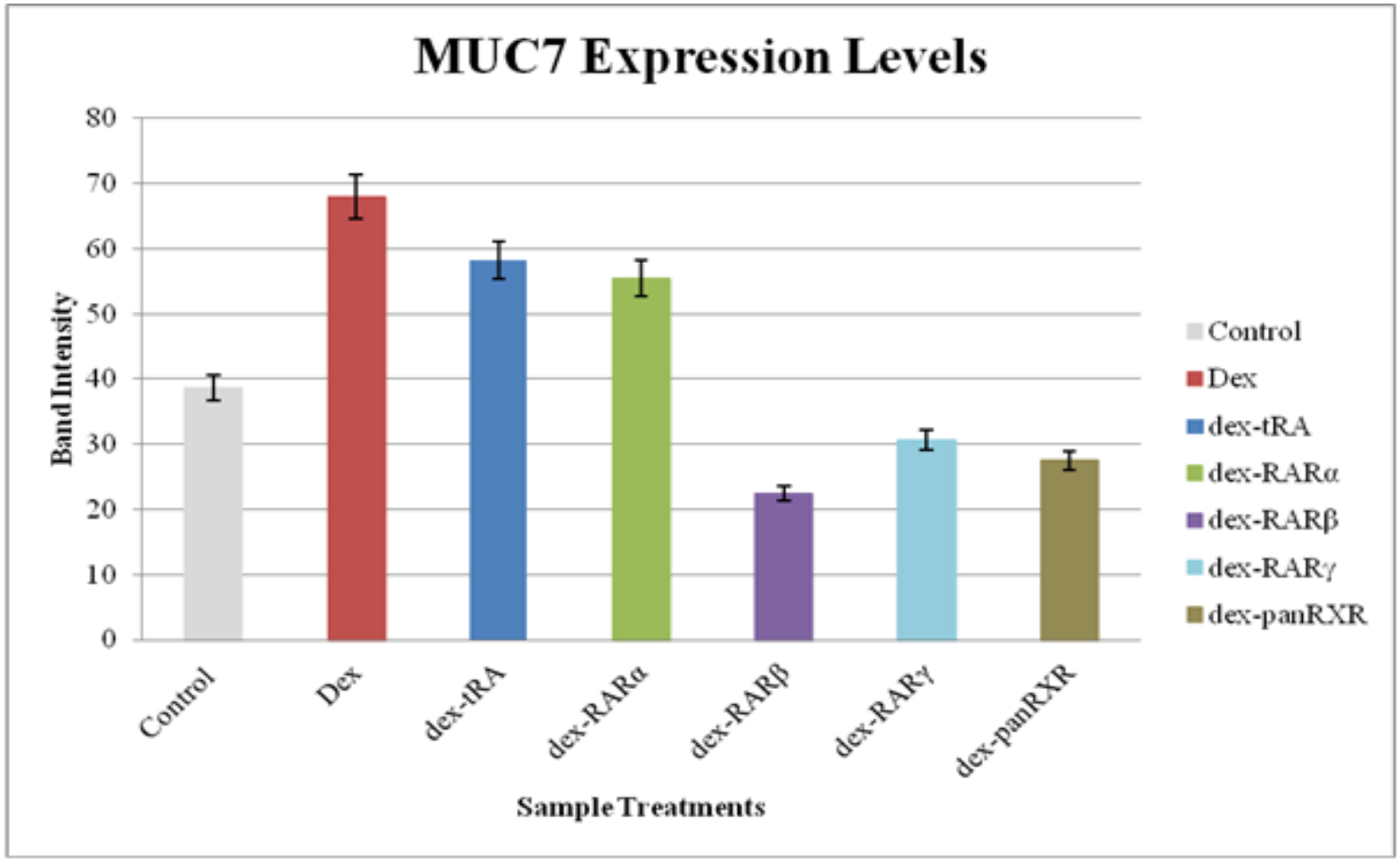

Fig 4.3.8d: Average measurements of MUC7 expression from homogenates of varying retinoid ligands. Dex-tRA

4.3.4: DISCUSSION: The main function of secreted mucins is their involvement in mucosa protection. Their cellular and tissue distribution are altered in pathological respiratory conditions and therefore are a vital component when diagnosing airway diseases. As in all cases of disease, it is important not only to focus on what goes wrong in chronic airway diseases associated with mucus hypersecretion, but also in the normal innate immune defence role that mucus (mucins) play in healthy tissues. For a long time, mucus hypersecretion was largely neglected as a clinical feature of COPD, but now it is a recognised important clinical characteristic, particularly for the role of mortality and morbidity associated with this disease. The severity of mucus hypersecretion in COPD patients can range from mild secretions to hospitalisation which if not treated, eventually leads to respiratory failure.

The aim of treating mucus hypersecretion is normally to alter rheological properties of bronchial mucus production; in affect, to reduce airway obstruction; to enhance mucociliary clearance and to promote expectoration (Kim 1997). There are strategies that have been put in place to reduce hypersecretion. Firstly to inhibit mucus hypersecretion by using pharmacological agents to stimulate ciliary activity and improve mucociliary interactions and last, to enhance coughing to enable mucus clearance by changing the rheological properties of mucus, again, by pharmacological means or by physical movement from smaller to larger airways from where it can be eliminated by coughing (Kim. 1997).

Studies have shown that retinoid ligands can inhibit the expression of proinflammatory processes, implicating them as regulators of immune and

inflammatory responses. This implies that retinoid ligands possess anti-inflammatory properties that may prove useful in the treatment of inflammatory lung disease exasperated by mucus hypersecretion.

Exposing mice to dexamethasone resulted in goblet cell metaplasia and hyperplasia, leading to mucin overproduction and mucus hypersecretion in postnatal lungs. We did not examine effects dexamethasone may have impacted on inflammatory mediators such as cytokines and neutrophil elastase, but it is well documented that these inflammatory agents contribute to excess mucin synthesis and mucus secretion in airways, so we can summarise that the same effect may be the reason for an increase of mucin expression that occurred in dexamethasone lungs.

Exposing mice with dexamethasone induced goblet cell hyperplasia in bronchial epithelium and upregulated MUC2, MUC5B and to a lesser extent MUC7 protein expression in postnatal lungs. These changes were associated with airway inflammation. From the data provided by this investigation, it is plausible that lung insult created by dexamethasone causes mucus hypersecretion by promoting an increase in muc protein expression, in particular MUC2 and MUC7 which are two muc proteins associated with airway disease. During chronic airway disease, upregulation of mucin expression in response to insults is known to be exaggerated and prolonged, which could in itself be a direct consequence of regulatory variation.

Introducing retinoid ligands to dexamethasone treated lungs reduced goblet cell hyperplasia and airway occlusions due to mucus hypersecretion.

Data suggests that tRA and RARα may have important roles in regulating processing related to airway mucus hypersecretion and airway hyperresponsiveness, indicating that vitamin A and its ligand show potential as targets to develop treatments for COPD/emphysema. Previous studies including chapter 3.3 have shown that deficiency of vitamin A is associated with altered mucus expression and that a reduction of this vitamin can attenuate COPD *in vivo* experimental models.

These results suggest that retinoids are required for proper lung maturation and response to injury. It would be interesting to explore whether retinoid induction to injured lungs have an essential role in the pathway through which monocytes/macrophage-derived micro-particles activate cytokines such as NFκB and ultimately induce the upregulation of proinflammatory mediators in postnatal lungs and whether RARα has the ability to downregulate proinflammatory protein expression.

4.3.5: SUMMARY. Mucus hypersecretion is therefore a complex pathologic process that involves the mechanisms that are responsible for hyperplasia and degranulation of mucus-secreting cells and the attraction of inflammatory cells. Mucins are an important component of host defence, but they represent an important cause of airway obstruction when secreted in excess. Whether goblet cells or

submucosal gland cells are the principal sources of mucins in airway secretions in COPD/emphysema or in other airway diseases is not known. In disease states, it is likely that the relative contribution of goblet cells and gland cells varies according to airway level, disease severity, and disease type. In COPD patients, it is clear that goblet cells contribute mucins to airway secretions and that retinoids are involved in the regulation of mucus hypersecretion.

CHAPTER 5

GENERAL DISCUSSION

5. GENERAL DISCUSSION

5.1: EPIDEMIOLOGY. Early diagnosis and treatment can markedly slow the decline of lung function and hence lengthen the period in prolonging the patient's life. An estimated 3 million people have COPD in the UK, although for around 2 million of this group remain undiagnosed. According to healthcare calculations, in the UK alone, the economic burden is around £1.2billion per annum and is steadily rising. This includes direct healthcare costs and factors such as lost income tax, payment of state benefits and productivity loss due to COPD. These calculations are based on the current age of retirement; if this is increased then the economic impact will inevitably rise (Fig 5.1).

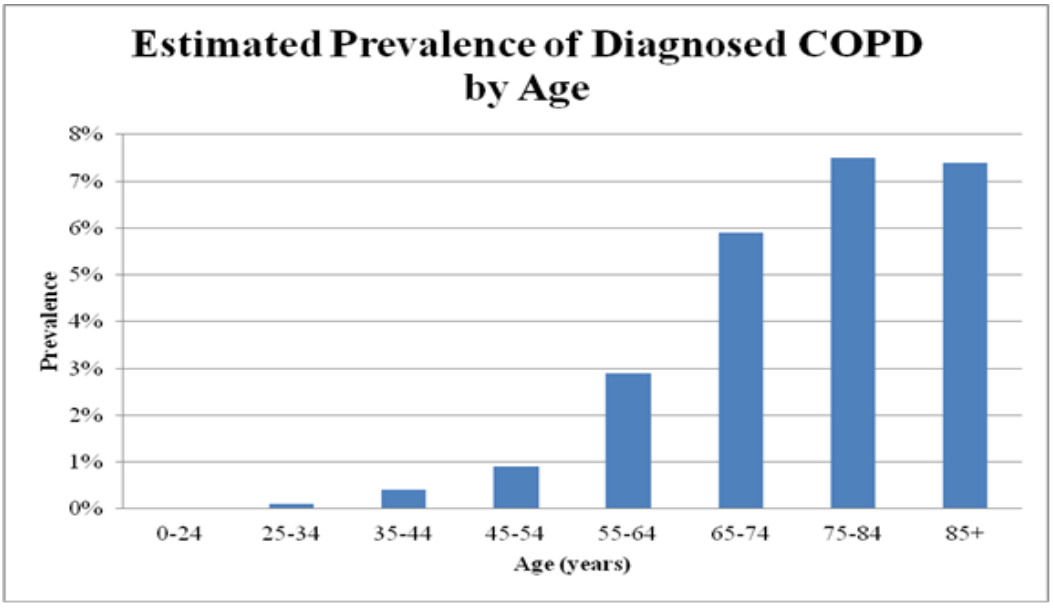

Fig 5.1: The prevalence of COPD is influenced by age as indicated in the figure above. The diagnosed prevalence for individuals between the ages of 45-54 years is <1% increasing to >5% for individuals aged 65 years and older. National costing report: chronic obstructive pulmonary disease. *niceclinicalguidelines101.org.uk. 2011*

5.2: ADVANCES IN RESEARCH. Most research on COPD has largely focused on effects of environmental factors such as initiators and causative agents, with little attention given to underlying or predisposing conditions. Despite the recognition of COPD as a multi-component disease, exacerbations are defined only in terms of respiratory symptoms: worsening of dyspnoea, cough and/or sputum production that is acute in onset and may warrant a change in regular medication regiment (Pauwels et al, 2001, Rodriguez-Roisin 2000).

There is no doubt that management of COPD/emphysema has greatly improved with the introduction of more effective treatments and the use of non-pharmacological interventions, such as pulmonary rehabilitation and non-invasive ventilation, but due to an increasing number of patients with COPD and indeed those dying from the disease, there is a pressing need for the development of new therapies especially as existing treatments are unable to reduce the progression of the disease (Barnes et al. 2005, Rennard 2004, Sutherland et al. 2004). New therapies could give rise to existing drugs such as β2-agonists and anticholinergic drugs which help with breathing problems by dilating the airways in the lungs (bronchodilators) and protect the airways from spasms that cause the narrowing of airways as well as reducing mucus hypersecretion.

There have been important advances in the current understanding of the cellular and molecular biology of COPD and through this knowledge, newer therapies are being developed for COPD specifically targeting inflammation of which is considered a major feature of this disease as it is presumed that it is the inflammatory processes which lead to structural changes occurring in COPD lungs and hence the scientific

rationale for the development of anti-inflammatory drugs, but in order to decipher which drug(s) are most effective, research has employed the use animal models (Fig 5.2 Barnes et al. 2005, Barnes et al. 2003, Barnes et al. 2002, Barnes et al. 2004).

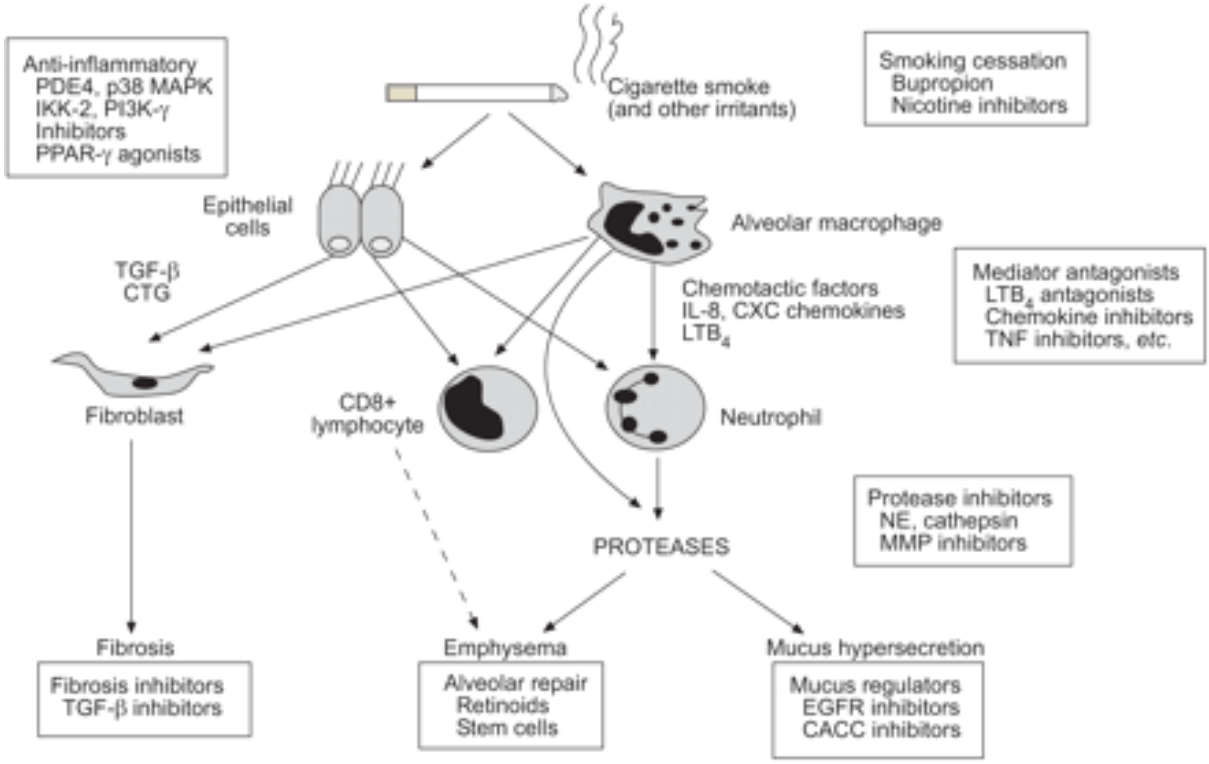

Fig 5.2: Targets for COPD therapy based on the current understanding of the inflammatory mechanisms. Cigarette smoke and other irritants activate macrophages in the respiratory tract that release neutrophil chemotatic factors, including interleukin (IL-8) and leukotriene B4 (LTB4). These cells then release protease that break down connective tissue in the lung parenchyma resulting in emphysema and also stimulates mucus hypersecretion. *Barnes et al. 2005. Eur Respir J. 25: 1084-1106*

5.3: MURINE MODELS OF AIRWAY DISEASE. There are both pros and cons of using animal models instead of human clinical studies. Murine models especially, have the advantage of short experimental cycles and are easy to study over time in a controlled environment. They allow for convenient specimen collection and a broad range of different animal models allow for investigation of mechanistic pathways for many diseases. Another advantage is that they are easy to breed and to house. The disadvantage is that sometimes it is difficult to make direct comparisons with human diseases, which reveals significant differences in sensitivity between murine and humans. For example, mice and rat lungs are considerably different in structure from that of the human lung. The airways of humans are unique, with far more branches than in other species, especially mice and rats and the outcome of specific treatments in both species is not necessary the same in humans. However, the obvious advantage of being able to exam systemic affects, have driven us to further develop the methods and protocols to improve the similarities to human diseases.

5.4: ROLE OF VITAMIN A IN AIRWAY DISEASE. The role of vitamin A in structure, function and maintenance of the lung is one of profound importance and therefore has had extensive research on these topics. In its active form, RA controls regular differentiation as ligands for RAR and RXR and is involved in the integration (gap junction formation) of cell formations. Vitamin A plays a substantial role in the respiratory epithelium and the lung. In VAD, the incidence of respiratory diseases is considerably increased and repeated respiratory injections can be influenced therapeutically by vitamin A supplementation (VA). In addition to the importance of VA for lung function, VA is also responsible for the development of

many tissues and cells as well as for embryonic lung development. Studies have shown that control occurs by different expressions of retinoid receptors as well as by time-dependent changes of the VA metabolism respectively via cellular VA binding proteins (CRBP: cytoplasmic retinoid binding protein; CRABP: cytoplasmic retinoid acid binding protein).

VA (retinol) and RA play a crucial role in lung development during embryogenesis; in terms of interactions with the genome, RA appears to be the most active of the retinoids. RA affects many aspects of lung development ranging from branching morphogenesis to structural remodelling during alveolarization. VA may also be involved in surfactant synthesis as VAD during gestation reduces the expression of surfactant protein A, B and C in foetal rat lungs. The role of RA in alveolarization and lung regeneration has received much attention. These effects are apparently mediated by elastin synthesis; endogenous retinoid production increases tropoelastin expression in vitro, whereas VAD during pregnancy reduces elastin staining in foetal lung. There is considerable interest in the possibility that RA treatment may be able to regenerate alveoli in human adult emphysematous lungs. As preterm infants can be VAD, they are often supplemented with VA to reduce oxygen requirements and treat lung injury, presumably by enhancement of alveolarization.

Malnutrition is a major problem, not only in developing countries, but also in westernised countries where there are pockets of poverty. Nutritional restriction or deficiency in adults has long been known to have adversely affect lung function and structure via alternations in pulmonary surfactant, respiratory control or respiratory muscle function. Poor nutrition can lead to the breakdown of structural proteins in

the lung which may exacerbate COPD. Poor nutrition is also known to impair immunity of lungs. We have shown in our VAD postnatal rat lung leads to the loss of airway structures, namely the thinning and fewer alveoli as well as the development of squamous metaplasia of the bronchial epithelia. However, this affect is only achievable if chronic as demonstrated in chapter 3.1 where we examined morphology and morphometric parameters. We demonstrated emphysematous-like features are achievable in VA depleted lungs of postnatal rats. Restoration to normal histological state occurs after VA repletion, which in experimental murine models has been shown to confer emphysematous-like features. Serious clinical manifestations of VAD in children create an increased risk of respiratory infection which is associated with changes in the respiratory epithelium (McDowell et al. 1990).

The processes underlying nutritional emphysema remains unclear, but components of lungs most affected by postnatal starvation is the alveolar septum. Nutritional emphysema maybe largely due to the breakdown of structural proteins of the septum, in particular, elastin and collagen, however, starvation must be severe to induce these changes. In the rat, it has been shown that starvation causes a reversible reduction in the activity of lysyl oxidase is involved in post-transitional cross-linking of elastin and collagen. Elastase treatment of rat lungs induces a form of emphysema that more closely resembles conditions in humans than starvation suggesting that elastin breakdown may be involved in the human disease. Of interest it has shown that nutritional emphysema in postnatal animals is reversible, caloric restriction in mice reduce the number of alveoli and alveolar surface area, as occurs in human emphysema, but re-feeding fully reverses these changes. This suggest that alveolar

walls are able to regenerate following their destruction due to deficient nutrition in murine animals.

Up to now, the use of retinoids in clinical trials has been limited because of their pharmacological and side effects, as it has been well documented that excess amounts of VA have teratogenic effects, embryonically, as well as having a role in osteoporosis, but the mechanisms through which retinoids restore emphysematous features are gradually being elucidated. Their complexity results from the large number of proteins involved in cell differentiation and proliferation (Blomhoff 1994, Bogos et al. 2008, Gorodischer 1993, Eskild et al. 1994, Penniston et al. 2006).

5.5: AIMS OF PROJECT. For the overall project, we wanted to assess therapeutic effects of ligands in retinoid-selective agonists in lungs administered with dexamethasone at a dosage of 4mg/kg to induced emphysema and to show that VA in the form of these naturally occurring and synthetic forms are important for the continuation of structural function and maintenance of the respiratory system, postnatally. To assess the role of retinoids in postnatal lungs, we used a nutritional approach. By generating postnatal VAD rats from the age of 3 months we determined the function of retinoids in these lungs. We demonstrated VAD rat lungs were morphologically and morphometrically different from age and sex-match control rat lungs. The alveolar septal walls of VAD rat lungs appeared thinner, with fewer and larger alveoli. There was a reduction in lung volume and evidence in alveolar enlargement i.e. emphysema. Results from morphometric analysis of VAD demonstrated a progressive increase in L_m, (a parameter widely used to evaluate the

severity of emphysema in animal models), from 6 months to 1 year animal groups compared with control groups. To demonstrate restoration of injured lungs induced by dexamethasone, we used the same L_m parameter we used for the examination of VAD rat model. There is evidence from animal models that exposure of the developing lung to high concentrations of dexamethasone inhibits alveolus formation and decreases lung surface area. We were able to demonstrate this feature in our postnatal mouse model (chapter 4.1). As expected, we found that both airspace areas and airspace volume density, L_m values too had greatly increased when comparing with control groups and that both model groups were significantly different $p<0.05$ from their control counterparts showing a shift in airway distribution, thus underlying structural changes.

There are concerns of this parameter regarding its properties to assess pulmonary emphysema quantitatively partly relating to the fact that the reference volume is frequently not considered, making its interpretation difficult (Knundsen et al. 2010, Hsia et al. 2008, Mitzner 2008, Weibel et al. 2007). Moreover, this parameter involves both alveolar space and alveolar duct space taken together. In addition, because it is only a mean, it cannot reflect the heterogeneity of pathological alternations, a typical feature of emphysema (Knunsden et al. 2010, Ito et al. 2004, Russi et al. 1999). Nevertheless, using this parameter we were able to determine that there were no differences in the mean lung volumes associated with exposure to dexamethasone or treatment with the retinoid ligand RARβγ or panRXR. They were unable to reverse dexamethasone effects on airspace size, airspace volume or the increase in vascularisation, but differences were found in airspace size and volume with tRA and RARα in comparison with dexamethasone animal groups, although

vascularisation appeared to show an increase with tRA animal groups in comparison with control groups. Similarly dexamethasone-treated animal displayed a decrease. RARγ and panRXR did not however reverse the dexamethasone effect on secondary alveolar structure. They were unable to alter airspace size or airspace volume density in the lungs of animals exposed with dexamethasone.

Overall morphological and morphometric data suggest that inhibition of septal formation may occur via different mechanisms with dexamethasone accounting for the ability of RA and retinoids to mitigate the effects of dexamethasone in alveolarization in postnatal animals. It is possible that the dose chosen for retinoid ligands throughout this study was not optimal to ameliorate the effects of dexamethasone on lung alveolarization. In these studies, the dose and routine of administration for tRA and retinoid ligands and dexamethasone were identical to those used by Hind and Maden (2004) in which tRA attenuated the reduction in alveolar number and body mass-specific surface area caused by dexamethasone administration

VA also appears to be involved in protecting the lung from dexamethasone injury. Our data indicates that after the addition of these retinoids, some (RARα and RARγ) efficiently reversed the effects of dexamethasone damage to the lung structure signifying, as have many experimental designs have, that these retinoids are positive regulator of restorative respiratory structures and that retinoids such as RARβ provide a very poor performance on reversing the effects of dexamethasone, thus signifying this retinoid as a negative regulator of restorative respiratory structures.

Along with tRA, RARα and RARγ showed an increase number of alveoli, but not total surface area of damaged lungs which may suggest that a prolonged treatment, rather than an increase in dose of these retinoids may completely restore alveolar structure.

Because retinoids may stimulate antioxidant defences and act as antioxidants, they may protect the lungs from damage by oxygen free radicals generated through direct exposure and by inflammatory cells. All of these findings are consistent with clinical observations of the therapeutic benefits of VA supplementation on postnatal lungs, but do not assess the effects of retinol on dexamethasone-induced changes on lung morphology such as those seen in BPD.

5.6: MUCUS HYPERSECRETION. Mucus production itself is troublesome to many patients with COPD, yet little is known regarding the mechanisms of mucus hypersecretion, the benefits and risks associated with increased mucus production, or the means whereby mucin secretion, its composition and mucociliary clearance might be therapeutically regulated. Therefore, research is needed to expand our understanding of molecular and cellular mechanisms of mucus metaplasia and excess mucus production. Studies of submucosal glands of the small airways are especially needed because these glands are probably most important in COPD and have

attracted relatively little research interest. In the classical phenotype of chronic bronchitis, mucus hypersecretion is the key presenting symptom and its contribution to airflow obstruction and has been the subject of debates for a long time (Prescott et

al. 1995, Cox 1972). However, results of several recent epidemiological studies have shown an association between mucus hypersecretion and patients with COPD. In a 22 year mortality survey of 1061 working males, respiratory mucus hypersecretion was found to constitute more than a negligible public health problem (Cerveri et al. 2010, Vesbo et al. 2006). In the Copenhagen City Heart Study, chronic mucus hypersecretion was found to be significantly and consistently associated with both an excess in FEV1 decline and an increase of subsequent hospitalizations due to COPD (Cerveri et al. 2010, Melton 2002). This topic has subsequently been the subject of a Novartis Foundation symposium, which quoted these epidemiological studies and concluded that dealing with inflammation in COPD patients might not be sufficient enough and that clearing mucus from the airways might provide a better opportunity to treat the inflammation and offer relief to the patient (Cerveri et al. 2010, Vestbo et al. 2002). The hypothesis that inflammation accounts for all features of pathobiologcal processes involved in alveolar destruction and airway remodelling in COPD slightly over simplifies the overall polymorphic and multi-factorial nature of inflammation in COPD and consequently, has led to major therapeutic advances in the treatment of COPD (Cerveri et al. 2010). Several observations, particularly related to the progressive nature of COPD, raise some questions that have not been adequately addressed such as; why does it take decades for the disease to develop? Why do some patients experience a rapid decline in lung function while others live for a long time with stable airflow obstruction? Why does inflammation persist despite smoking cessation? Why do corticosteroids have little impact on the natural history of COPD, except for exacerbations? (Cerveri et al. 2010).

Mucins are extremely heterogeneous in their oligosaccharide structure; therefore its population can be divided into subsets depending on which epitope of mucin is to be measured. We used histochemical, immunohistochemical and western blots to analyse stimulatory effect of experimentally injured lungs and injured lungs undergoing regeneration on mucin production. Both VAD and dexamethasone animal groups showed acidic glycoproteins being greater in secretion than neutral glycoproteins. These groups also showed a significant increase in the amount of acidic glycoproteins secreted compared with control animals.

There are a number of questions that need to be addressed as regards to the data obtained with this investigation and the mucins used. Firstly, the role of individual MUC protein products are not entirely clear, as it is possible that many subsets of mucins may exist in terms of different biological glycosylation and sulfation, which may have different biological functions either alone or in association with its accessory proteins. For example, from the data obtained from our mucus hypersecretion, we concluded that an increase in certain airway mucins that might be beneficial to the airway defense and that other types may be detrimental to mucociliary clearance simply from the standpoint of the rheological properties that is determined by the structure of mucins. Thus it was extremely important to try to ascertain the role of individual airway mucin proteins in injured and regenerative lung. Like airway challenged diseases, dexamethasone showed an increase in all muc proteins used, especially in muc proteins not normally present in airway epithelia. What was interesting about this study was that dex-tRA and dex-RARβ were both negative for MUC5AC and both showed low protein expression of MUC5B. As far as we are aware, this response has not been noted in any other muc

protein study, repeats of these two protein perhaps need to be reassessed to confirm the results presented, but it could be that many differences in the cells of origin of mucus cells depend upon the nature of the injury that induces the mucus metaplasia and that tRA and RARβ are not involved in MUC5AC and MUC5B pathway. We also noted an increase in basal cell proliferation after VAD and dexamethasone challenge. We speculated that the increase in basal cell-containing epithelia may allow an early and rapid response of the airway epithelia to an injury.

Even if mucus production appears to be particularly important in COPD, its precise role in the development and progression of the disease is not completely understood. Hogg et al. have proposed that mucus could increase in the small airways, either because it is produced in excess by goblet cells stimulated by neutrophil elastase or oxygen free-radicals or because it is unable to flow out as a result of impaired mucus clearance (Cerveri et al. 2010, Hogg et al. 2007). The mucus could also stick to airway walls, thus contributing to luminal occlusion and lung function decline because of its viscosity.

The most prominent effect of retinoid ligand treatment obtained in these studies was the reduction of mucus hypersecretion. Expression of airway mucus proteins, MUC5AC and MUC5B as well as MUC2 and MUC7 was most noted in tRA and RARα treated lungs. There was also a more balanced population of acidic and neutral glycoproteins in these treated lungs suggesting that these two groups of treated lungs are involved in the regulation of mucociliary clearance. The mechanisms by which RARα alters mucin expression are unknown, however, the

lungs containing both tRA and RARα bind to specific DNA sequences near promoters of responsive genes and exert effects on transcription.

Although information concerning protein activation by RA binding to RAR is limited, RA has been demonstrated to alter protein expression. Therefore it is plausible that changes in expression of mucin protein regulated by tRA and RARα are major factors in alterations of mucus hypersecretion and that a decrease in mucin expression restores some lung function, resulting in greater survival of dexamethasone lung injury.

Although the doses used for all retinoid ligand treatment during these studies prevented impairment of septation/induced alveolar formation, progenitor cell response or mucus hypersecretion in postnatal mice lungs, further investigations relating to these studies are warranted.

5.7: PROGENITOR CELLS IN REGENERATION. Prior animal studies of allergic lung disease have largely focused on the murine bronchiole epithelium and suggested that Clara cells are the cells of goblet cells after an allergen challenge. A decrease in the number of Clara cells accompanied by an increase in the number of mucus secreting goblet cells in the bronchiole epithelia of OVA-challenged mice suggest that in this region of the airway, Clara cells convert to mucus cells (Saganta et al. 2012, Reader et al. 2003). Furthermore, using the OVA mouse model of airway disease, Evans et al. Observed mucin expression in a subset of Clara cells, again suggesting that Clara cells serve as progenitors of goblet cells. These observations were also consistent with another study showing that mucus cells produced in the

airways of OVA-treated mice include many ultrastructural characteristics of Clara cells documented by electron microscopy (Saganta et al. 2012, Evans et al. 2004, Hayashi et al. 2004). However, this study could not exclude the possibility that there was also contribution of some small airway ciliated cells to goblet cell pool. This study also did not address the cell of origin of mucus cells in pseudostratified basal cell-containing airway epithelium. We noted that a reduction of Clara cells was not achieved with both VAD and dexamethasone lungs, instead there was not only a slight increase, but a much reduced level of proliferative cells, which suggests that Clara cells present in both lung models maybe variants of the cell and may perhaps function differently. How the function of these cells are altered was not investigated, but may be an interesting topic to examine along with possible functional changes in the retinoid treated lungs as Clara cells were seen to proliferate in some retinoid treated lungs. Although proliferation of the bronchiole epithelium was exclusively in the basal cell in dexamethasone lungs, the increase of mucus-secreting goblet cells suggests that mucus cells directly arise from Clara or basal cells that themselves will not replicate at any time during the administration of dexamethasone before they and Clara cell populations are meant to replenish non-replicating basal and/or Clara cells that may be converted to ciliated or mucus cells. It would be interesting to assay whether replicating progenitor cells are necessary to fuel a continual supply of new mucus cells in chronic models of COPD. The results presented also suggest that dexamethasone and VAD exert inflammatory properties through different mechanisms. Dexamethasone and VAD had a profound effect on ATII cells where numbers were much reduced due to a reduction of alveoli epithelia and perhaps due to the process of differentiating into damaged ATI cells. Nevertheless, it is clear that the transcription factor Nkx2.1 still has a profound role in the regulation of ATII

cells and is equally affected by airway injury, because when there is a decrease in NKx2.1, there is also a decrease in SP-C-positive ATII cells and when there is an increase of Nkx2.1, then there is an increase in SP-C protein. Both VAD rat and dexamethasone mouse lungs led to a reduction in the pulmonary surfactant content, reduced lung compliance and a reduction of lamella bodies in ATII cells these effects may be due to a reduced availability of the substrates necessary for the syntheses of surfactant proteins. In adult rats, caloric restriction caused by a reduction in pool size of pulmonary surfactant proteins.

The stimulation of alveolar regeneration is an exciting possibility for disease-modifying therapy of COPD. Fundamental advances in this area are likely to derive from animal studies of alveolar development in the late foetal and postnatal periods. Such research might include gene expression and proteomic analysis of the developing lung, studies of the regulation of expression of relevant genes, studies of the coordination of vascularisation and lung development and repair, use of transgenic mice to evaluate the role of specific growth factors in the lung and investigations of the mechanisms whereby toxins impair lung growth. In addition, studies of alveolar regeneration in adults of multiple species are needed to determine the capacity of mature lungs for alveolar re-growth and the conditions under which alveolar regeneration can occur.

Finally, the development of new animal models of COPD is important for hypothesis testing regarding the pathogenic mechanisms of COPD. Topics of special interest include the biochemical basis of lung growth, damage and repair; the

necessity and sufficiency of specific inflammatory and mucus pathways for the development of small airways disease and the reversibility of lung damage. Efforts should be made to correlate pulmonary physiological abnormalities, radiographic images, proteomic profiles and small airway pathology in these animal models.

5.8: SUMMARY. The classical approach to COPD has encompassed different disorders in a unique entity under a variety of imprecise definitions and diagnostic criteria. The current definition fails to take into account the heterogeneity of the disease and does not reflect the different phenotypic distinctions which may be associated with differing natural histories, clinical features and response to treatments. The problem with disregarding the heterogeneity of COPD is that not all individual patients respond to a given treatment and therefore the opportunity to optimize therapy may be missed. Studies have identified different clusters of COPD patients based on both clinical and physiological characteristics. These include exacerbations, rapid decline of lung function, airway hyperresponsiveness, impaired exercise tolerance and emphysema. This large heterogeneity of COPD phenotypic distinctions may explain the failure of some large clinical trials to achieve statistical significant effects on primary outcomes.

Whether there is alveolar destruction, disruption of alveolar repair or a failure of further of alveolar formation is not yet known, but it seems likely that this model will contribute to the understanding of the pathogenesis of COPD.

CHAPTER 6

FUTURE DIRECTIONS

6.1: INTRODUCTION. Although the results present in this thesis have provided some insight into the many roles of retinoids in postnatal lung regeneration, these findings have also opened further lines of research which are worth pursuing. The ultimate goal of respiratory research is the hope that the gas exchanging surface area can be manipulated in the human lung. One such parameter relating to respiratory disorder and repair are rheological features of muc proteins and the ultimate effect they impose on breathing. As previously mentioned throughout this thesis, mucins are essential for the protection of internal epithelial surfaces. Molecular responses involving mucin production and secretion in our VAD and dexamethasone may be in response to infectious agents in the airway and may ultimately effect rheology and be a contributor to lung malfunction.

Since mucus was first implicated in COPD/emphysema, a number of hypothesis have been advanced to explain the characteristics of thick mucus. Like CF, it was thought that mucus was abnormally synthesised, but investigations have uncovered little data to indicate this to be true. Changes in sulfation and fructose to sialic acid ratios may be a factor involved in changes of mucus (mucin) composition in both dexamethasone and VAD animal models in relation to an increase of inflammation and infection, aspects of the study which is worth pursuing.

Mucins themselves tends to aggregate and immobilise, thereby obstructing ductal and luminal passages. This may imply that it is not that the mucin produced is abnormal, but the manner and conditions under which mucins are released results in the organs pathological fate (Quinton. 2008).

It would be interesting to examine whether mucus secreted by COPD/VAD animals is thick and sticky like mucus secreted in CF patients. It is generally believed that in CF, the “thickness” and “stickiness” of mucus is due to dehydration. Two general concepts have been devised for this theory, the first being from observations in the airways and airway-derived cells, that fluids are hyper-absorbed by the epithelium into which mucins are secreted and therefore is not adequately hydrated (Quinton. 2008, Boucher. 2007). It is thought that this rationale shared with normal or CF phenotypes, but this does not necessarily mean that this rationale is not true for COPD/emphysema airways or for the explanation occurring in the animal models used in this thesis. The second explanation/theory of dehydration is ascribed to hypo-secretion of fluids (Quinton. 2008, Trout et al. 1998, Joo et al. 2006). The problem with this theory is that hypo-secretion assumes that once mucins are secreted, are without access to fluid which seems unlikely as secretory epithelium affected organs are highly permeable to water (Quinton. 2008). Therefore there must be another factor involved in mucus hypersecretion and change in mucin composition. Quinton. 2008 has proposed that bicarbonate secretion (HCO_3^-) may be the cause of mucus dehydration (thickness and stickiness) and mucin compositional change and may also be occurring in the animal models used in this thesis. The concept behind this is thus; gel forming mucins protect the apical surfaces of epithelial tissues. These molecules are compacted and stored intracellularly as granules with high concentrations of calcium ions (Ca++). These cations shield repulsive forces of fixed negative charges on mucins to prevent their expansion in forming mucus gels. Ca++ also forms electrostatic divalent bridges between fixed negative charges that further stabilise mucins and oppose expansion (Quinton. 2008, Perez-Vilar et al. 2005). Once the granule is released, HCO_3^- is

critical to sequester Ca++ and H+ away from mucin anions by complicating with them, i.e. HCO_3^- and its equilibrium form CO_3^{2-}, react with condensed mucins to form H_2CO_3, $CaHCO_3+$ and $CaCO_3$ and reduce the activity of Ca++ and H+ in the mucin solution. Once unshielded, the electrostatic anionic forces rapidly expand the mucin macromolecules. There are no other anions of significance in secreted fluids capable of effectively complicating these cation. The only other significant anion, Cl- cannot buffer H+ or chelate Ca++ because both HCl and CaCl2 are highly dissociable (Quinton. 2008). Quinton proposes that in CF, the lack of HCO_3^- in the extracellular medium of mucins impairs calcium removal, prevents normal mucin expansion and promotes stasis of mucus in the ducts or on the luminal surfaces of affected organs. This action could be happening in dexamethasone/VAD lungs and is worth pursuing. How is retinoid ligands involved in the reversal effect of the HCO_3^- theory, if true in these models and thus in human lungs? Are any of the retinoid ligands used throughout this thesis able to reverse the thickness and stickiness by restoring levels of HCO_3^-.

Adamson, I. Y. and D. H. Bowden (1974). "The type 2 cell as progenitor of alveolar epithelial regeneration. A cytodynamic study in mice after exposure to oxygen." Lab Invest **30**(1): 35-42.

Adamson, I. Y. and D. H. Bowden (1975). "Derivation of type 1 epithelium from type 2 cells in the developing rat lung." Lab Invest **32**(6): 736-745.

Alon, T., I. Hemo, A. Itin, J. Pe'er, J. Stone and E. Keshet (1995). "Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity." Nat Med **1**(10): 1024-1028.

Amy, R. W., D. Bowes, P. H. Burri, J. Haines and W. M. Thurlbeck (1977). "Postnatal growth of the mouse lung." J Anat **124**(Pt 1): 131-151.

Aoshiba, K., N. Yokohori and A. Nagai (2003). "Alveolar wall apoptosis causes lung destruction and emphysematous changes." Am J Respir Cell Mol Biol **28**(5): 555-562.

Auten, R. L., R. H. Watkins, D. L. Shapiro and S. Horowitz (1990). "Surfactant apoprotein A (SP-A) is synthesized in airway cells." Am J Respir Cell Mol Biol **3**(5): 491-496.

Ayers, M. M. and P. K. Jeffery (1988). "Proliferation and differentiation in mammalian airway epithelium." Eur Respir J **1**(1): 58-80.

Balda, M. S. and K. Matter (2000). "Transmembrane proteins of tight junctions." Semin Cell Dev Biol **11**(4): 281-289.

Barnes, P. J., S. D. Shapiro and R. A. Pauwels (2003). "Chronic obstructive pulmonary disease: molecular and cellular mechanisms." Eur Respir J **22**(4): 672-688.

Bartalesi, B., E. Cavarra, S. Fineschi, M. Lucattelli, B. Lunghi, P. A. Martorana and G. Lungarella (2005). "Different lung responses to cigarette smoke in two strains of mice sensitive to oxidants." Eur Respir J **25**(1): 15-22.

Baybutt, R. C., L. Hu and A. Molteni (2000). "Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type II pneumocytes." J Nutr **130**(5): 1159-1165.

Belloni, P. N., L. Garvin, C. P. Mao, I. Bailey-Healy and D. Leaffer (2000). "Effects of all-trans-retinoic acid in promoting alveolar repair." Chest **117**(5 Suppl 1): 235S-241S.

Bohinski, R. J., R. Di Lauro and J. A. Whitsett (1994). "The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis." Mol Cell Biol **14**(9): 5671-5681.

Bolton, S. J., K. Pinnion, C. V. Marshall, E. Wilson, J. E. Barker, V. Oreffo and M. L. Foster (2008). "Changes in Clara cell 10 kDa protein (CC10)-positive cell distribution in acute lung injury following repeated lipopolysaccharide challenge in the rat." Toxicol Pathol **36**(3): 440-448.

Borok, Z., S. I. Danto, R. L. Lubman, Y. Cao, M. C. Williams and E. D. Crandall (1998). "Modulation of t1alpha expression with alveolar epithelial cell phenotype in vitro." Am J Physiol **275**(1 Pt 1): L155-164.

Borthwick, D. W., M. Shahbazian, Q. T. Krantz, J. R. Dorin and S. H. Randell (2001). "Evidence for stem-cell niches in the tracheal epithelium." Am J Respir Cell Mol Biol **24**(6): 662-670.

Bos, A. P., S. M. Hussain, F. W. Hazebroek, D. Tibboel, M. Meradji and J. C. Molenaar (1993). "Radiographic evidence of bronchopulmonary dysplasia in high-risk congenital diaphragmatic hernia survivors." Pediatr Pulmonol **15**(4): 231-234.

Bowler, R. P. and J. D. Crapo (2002). "Oxidative stress in airways: is there a role for extracellular superoxide dismutase?" Am J Respir Crit Care Med **166**(12 Pt 2): S38-43.

Bruno, M. D., R. J. Bohinski, K. M. Huelsman, J. A. Whitsett and T. R. Korfhagen (1995). "Lung cell-specific expression of the murine surfactant protein A (SP-A) gene is mediated by interactions between the SP-A promoter and thyroid transcription factor-1." J Biol Chem **270**(12): 6531-6536.

Brusselle, G. G., K. R. Bracke, T. Maes, I. D'Hulst A, K. B. Moerloose, G. F. Joos and R. A. Pauwels (2006). "Murine models of COPD." Pulm Pharmacol Ther **19**(3): 155-165.

Burgess, K. A., H. H. Hu, W. R. Wagner and W. J. Federspiel (2009). "Towards microfabricated biohybrid artificial lung modules for chronic respiratory support." Biomed Microdevices **11**(1): 117-127.

Burri, P. H. (1974). "The postnatal growth of the rat lung. 3. Morphology." Anat Rec **180**(1): 77-98.

Burri, P. H. (1985). "Morphology and respiratory function of the alveolar unit." Int Arch Allergy Appl Immunol **76 Suppl 1**: 2-12.

Burri, P. H. (2006). "Structural aspects of postnatal lung development - alveolar formation and growth." Biol Neonate **89**(4): 313-322.

Burri, P. H. and V. Djonov (2002). "Intussusceptive angiogenesis--the alternative to capillary sprouting." Mol Aspects Med **23**(6S): S1-27.

Burri, P. H. and M. Moschopulos (1992). "Structural analysis of fetal rat lung development." Anat Rec **234**(3): 399-418.

Busch, R. H., K. E. Lauhala, S. M. Loscutoff and K. E. McDonald (1984). "Experimental pulmonary emphysema induced in the rat by intratracheally administered elastase: morphogenesis." Environ Res **33**(2): 497-513.

Cantin, A. M., S. L. North, G. A. Fells, R. C. Hubbard and R. G. Crystal (1987). "Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis." J Clin Invest **79**(6): 1665-1673.

Cardoso, W. V. and J. Lu (2006). "Regulation of early lung morphogenesis: questions, facts and controversies." Development **133**(9): 1611-1624.

Cardoso, W. V. and J. A. Whitsett (2008). "Resident cellular components of the lung: developmental aspects." Proc Am Thorac Soc **5**(7): 767-771.

Cardoso, W. V. and M. C. Williams (2001). "Basic mechanisms of lung development: Eighth Woods Hole Conference on Lung Cell Biology 2000." Am J Respir Cell Mol Biol **25**(2): 137-140.

Cavarra, E., B. Bartalesi, M. Lucattelli, S. Fineschi, B. Lunghi, F. Gambelli, L. A. Ortiz, P. A. Martorana and G. Lungarella (2001). "Effects of cigarette smoke in mice with different levels of alpha(1)-proteinase inhibitor and sensitivity to oxidants." Am J Respir Crit Care Med **164**(5): 886-890.

Chailley-Heu, B., N. Chelly, M. Lelievre-Pegorier, A. M. Barlier-Mur, C. Merlet-Benichou and J. R. Bourbon (1999). "Mild vitamin A deficiency delays fetal lung maturation in the rat." Am J Respir Cell Mol Biol **21**(1): 89-96.

Chinoy, M. R. (2003). "Lung growth and development." Front Biosci **8**: d392-415.

Churg, A., S. Zhou and J. L. Wright (2012). "Series "matrix metalloproteinases in lung health and disease": Matrix metalloproteinases in COPD." Eur Respir J **39**(1): 197-209.

Chytil, F. (1992). "The lungs and vitamin A." Am J Physiol **262**(5 Pt 1): L517-527.

Chytil, F. (1996). "Retinoids in lung development." FASEB J **10**(9): 986-992.

Cilley, R. E., J. R. Wesley, J. B. Zwischenberger, R. E. Dechert and R. H. Bartlett (1987). "Metabolic rates of newborn infants with severe respiratory failure treated with extracorporeal membrane oxygenation." Curr Surg **44**(1): 48-51.

Clegg, G. R., C. Tyrrell, S. R. McKechnie, M. F. Beers, D. Harrison and M. C. McElroy (2005). "Coexpression of RTI40 with alveolar epithelial type II cell proteins in lungs following injury: identification of alveolar intermediate cell types." Am J Physiol Lung Cell Mol Physiol **289**(3): L382-390.

Cohn, L. (2006). "Mucus in chronic airway diseases: sorting out the sticky details." J Clin Invest **116**(2): 306-308.

Copin, M. C., M. P. Buisine, E. Leteurtre, C. H. Marquette, H. Porte, J. P. Aubert, B. Gosselin and N. Porchet (2001). "Mucinous bronchioloalveolar carcinomas display a specific pattern of mucin gene expression among primary lung adenocarcinomas." Hum Pathol **32**(3): 274-281.

Coraux, C., J. Roux, T. Jolly and P. Birembaut (2008). "Epithelial cell-extracellular matrix interactions and stem cells in airway epithelial regeneration." Proc Am Thorac Soc **5**(6): 689-694.

Coyne, C. B., T. M. Gambling, R. C. Boucher, J. L. Carson and L. G. Johnson (2003). "Role of claudin interactions in airway tight junctional permeability." Am J Physiol Lung Cell Mol Physiol **285**(5): L1166-1178.

Cutz, E. (1982). "Neuroendocrine cells of the lung. An overview of morphologic characteristics and development." Exp Lung Res **3**(3-4): 185-208.

D'Ambrosio, D. N., R. D. Clugston and W. S. Blaner (2011). "Vitamin A metabolism: an update." Nutrients **3**(1): 63-103.

D'Armiento, J., S. S. Dalal, Y. Okada, R. A. Berg and K. Chada (1992). "Collagenase expression in the lungs of transgenic mice causes pulmonary emphysema." Cell **71**(6): 955-961.

d'Hulst, D., J. Butterworth, S. Dale, T. Oaks and B. Matthews (2004). "Polypoid hyperplasia of the larynx misdiagnosed as a malpositioned laryngeal mask airway." Anesth Analg **99**(5): 1570-1572; table of contents.

Daly, H. E., C. M. Baecher-Allan, A. T. Paxhia, R. M. Ryan, R. K. Barth and J. N. Finkelstein (1998). "Cell-specific gene expression reveals changes in epithelial cell populations after bleomycin treatment." Lab Invest **78**(4): 393-400.

Daugherty, B. L., M. Mateescu, A. S. Patel, K. Wade, S. Kimura, L. W. Gonzales, S. Guttentag, P. L. Ballard and M. Koval (2004). "Developmental regulation of claudin localization by fetal alveolar epithelial cells." Am J Physiol Lung Cell Mol Physiol **287**(6): L1266-1273.

Daviskas, E., M. Robinson, S. D. Anderson and P. T. Bye (2002). "Osmotic stimuli increase clearance of mucus in patients with mucociliary dysfunction." J Aerosol Med **15**(3): 331-341.

Dawson, M. I. and X. K. Zhang (2002). "Discovery and design of retinoic acid receptor and retinoid X receptor class- and subtype-selective synthetic analogs of all-trans-retinoic acid and 9-cis-retinoic acid." Curr Med Chem **9**(6): 623-637.

de Santi, M. M., P. A. Martorana, E. Cavarra and G. Lungarella (1995). "Pallid mice with genetic emphysema. Neutrophil elastase burden and elastin loss occur without alteration in the bronchoalveolar lavage cell population." Lab Invest **73**(1): 40-47.

DeFelice, M., D. Silberschmidt, R. DiLauro, Y. Xu, S. E. Wert, T. E. Weaver, C. J. Bachurski, J. C. Clark and J. A. Whitsett (2003). "TTF-1 phosphorylation is required for peripheral lung morphogenesis, perinatal survival, and tissue-specific gene expression." J Biol Chem **278**(37): 35574-35583.

deMello, D. E. (2004). "Pulmonary pathology." Semin Neonatol **9**(4): 311-329.

Desai, T. J., F. Chen, J. Lu, J. Qian, K. Niederreither, P. Dolle, P. Chambon and W. V. Cardoso (2006). "Distinct roles for retinoic acid receptors alpha and beta in early lung morphogenesis." Dev Biol **291**(1): 12-24.

Desai, T. J., S. Malpel, G. R. Flentke, S. M. Smith and W. V. Cardoso (2004). "Retinoic acid selectively regulates Fgf10 expression and maintains cell identity in the prospective lung field of the developing foregut." Dev Biol **273**(2): 402-415.

Devereux, T. R. (1984). "Alveolar type II and Clara cells: isolation and xenobiotic metabolism." Environ Health Perspect **56**: 95-101.

Djonov, V. G., H. Kurz and P. H. Burri (2002). "Optimality in the developing vascular system: branching remodeling by means of intussusception as an efficient adaptation mechanism." Dev Dyn **224**(4): 391-402.

Dor, Y. and D. A. Melton (2004). "How important are adult stem cells for tissue maintenance?" Cell Cycle **3**(9): 1104-1106.

Duncan, T. E., J. B. Green and M. H. Green (1993). "Liver vitamin A levels in rats are predicted by a modified isotope dilution technique." J Nutr **123**(5): 933-939.

Elizur, A., T. L. Adair-Kirk, D. G. Kelley, G. L. Griffin, D. E. Demello and R. M. Senior (2008). "Tumor necrosis factor-alpha from macrophages enhances LPS-induced clara cell expression of keratinocyte-derived chemokine." Am J Respir Cell Mol Biol **38**(1): 8-15.

Engelhardt, J. F. (2001). "Stem cell niches in the mouse airway." Am J Respir Cell Mol Biol **24**(6): 649-652.

Evans, C. M., O. W. Williams, M. J. Tuvim, R. Nigam, G. P. Mixides, M. R. Blackburn, F. J. DeMayo, A. R. Burns, C. Smith, S. D. Reynolds, B. R. Stripp and B. F. Dickey (2004). "Mucin is produced by clara cells in the proximal airways of antigen-challenged mice." Am J Respir Cell Mol Biol **31**(4): 382-394.

Evans, M. J., L. J. Cabral-Anderson and G. Freeman (1978). "Role of the Clara cell in renewal of the bronchiolar epithelium." Lab Invest **38**(6): 648-653.

Evans, M. J., L. J. Cabral, R. J. Stephens and G. Freeman (1975). "Transformation of alveolar type 2 cells to type 1 cells following exposure to NO2." Exp Mol Pathol **22**(1): 142-150.

Farmer, L. J., L. Zhi, S. Jeong, W. W. Lamph, D. L. Osburn, G. Croston, K. S. Flatten, R. A. Heyman and A. M. Nadzan (2003). "Retinoic acid receptor ligands based on the 6-cyclopropyl-2,4-hexadienoic acid." Bioorg Med Chem Lett **13**(2): 261-264.

Farquhar, M. G. and G. E. Palade (1963). "Junctional complexes in various epithelia." J Cell Biol **17**: 375-412.

Fehrenbach, H. (2006). "Morphological quantitation of emphysema: a debate." J Appl Physiol **100**(4): 1423-1424.

Fernandez, A. L., M. Koval, X. Fan and D. M. Guidot (2007). "Chronic alcohol ingestion alters claudin expression in the alveolar epithelium of rats." Alcohol **41**(5): 371-379.

Folli, C., D. Descalzi, F. Scordamaglia, A. M. Riccio, C. Gamalero and G. W. Canonica (2008). "New insights into airway remodelling in asthma and its possible modulation." Curr Opin Allergy Clin Immunol **8**(5): 367-375.

Frankenberger, M., I. Heimbeck, W. Moller, S. Mamidi, G. Kassner, K. Pukelsheim, M. Wjst, M. Neiswirth, P. Kroneberg, D. Lomas, D. Halsall, P. Iadarola, A. Fertl, K. Haussinger and L. Ziegler-Heitbrock (2009). "Inhaled all-trans retinoic acid in an individual with severe emphysema." Eur Respir J **34**(6): 1487-1489.

Fregonese, L. and J. Stolk (2008). "Hereditary alpha-1-antitrypsin deficiency and its clinical consequences." Orphanet J Rare Dis **3**: 16.

Frey, G., E. Egli, B. Chailley-Heu, M. Lelievre-Pegorier, P. H. Burri, J. Bourbon and S. A. Tschanz (2004). "Effects of mild vitamin a deficiency on lung maturation in newborn rats: a morphometric and morphologic study." Biol Neonate **86**(4): 259-268.

Fujita, M., Q. Ye, H. Ouchi, N. Nakashima, N. Hamada, N. Hagimoto, K. Kuwano, R. J. Mason and Y. Nakanishi (2004). "Retinoic acid fails to reverse emphysema in adult mouse models." Thorax **59**(3): 224-230.

Furuse, M., K. Fujita, T. Hiiragi, K. Fujimoto and S. Tsukita (1998). "Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin." J Cell Biol **141**(7): 1539-1550.

Gardi, C., E. Cavarra, P. Calzoni, P. Marcolongo, M. de Santi, P. A. Martorana and G. Lungarella (1994). "Neutrophil lysosomal dysfunctions in mutant C57 Bl/6J mice: interstrain variations in content of lysosomal elastase, cathepsin G and their inhibitors." Biochem J **299 (Pt 1)**: 237-245.

Giangreco, A., E. N. Arwert, I. R. Rosewell, J. Snyder, F. M. Watt and B. R. Stripp (2009). "Stem cells are dispensable for lung homeostasis but restore airways after injury." Proc Natl Acad Sci U S A **106**(23): 9286-9291.

Gonzalez, R., Y. H. Yang, C. Griffin, L. Allen, Z. Tigue and L. Dobbs (2005). "Freshly isolated rat alveolar type I cells, type II cells, and cultured type II cells have distinct molecular phenotypes." Am J Physiol Lung Cell Mol Physiol **288**(1): L179-189.

Greene, K. E., J. R. Wright, K. P. Steinberg, J. T. Ruzinski, E. Caldwell, W. B. Wong, W. Hull, J. A. Whitsett, T. Akino, Y. Kuroki, H. Nagae, L. D. Hudson and T. R. Martin (1999). "Serial changes in surfactant-associated proteins in lung and serum before and after onset of ARDS." Am J Respir Crit Care Med **160**(6): 1843-1850.

Gross, I. (1990). "Regulation of fetal lung maturation." Am J Physiol **259**(6 Pt 1): L337-344.

Gross, P., E. A. Pfitzer, E. Tolker, M. A. Babyak and M. Kaschak (1965). "Experimental Emphysema: Its Production with Papain in Normal and Silicotic Rats." Arch Environ Health **11**: 50-58.

Grummer, M. A. and R. D. Zachman (1995). "Postnatal rat lung retinoic acid receptor (RAR) mRNA expression and effects of dexamethasone on RAR beta mRNA." Pediatr Pulmonol **20**(4): 234-240.

Guazzi, S., R. Lonigro, L. Pintonello, E. Boncinelli, R. Di Lauro and F. Mavilio (1994). "The thyroid transcription factor-1 gene is a candidate target for regulation by Hox proteins." EMBO J **13**(14): 3339-3347.

Gudas, L. J. (2012). "Emerging roles for retinoids in regeneration and differentiation in normal and disease states." Biochim Biophys Acta **1821**(1): 213-221.

Guerassimov, A., Y. Hoshino, Y. Takubo, A. Turcotte, M. Yamamoto, H. Ghezzo, A. Triantafillopoulos, K. Whittaker, J. R. Hoidal and M. G. Cosio (2004). "The development of emphysema in cigarette smoke-exposed mice is strain dependent." Am J Respir Crit Care Med **170**(9): 974-980.

Guo, Y., C. Martinez-Williams, C. E. Yellowley, H. J. Donahue and D. E. Rannels (2001). "Connexin expression by alveolar epithelial cells is regulated by extracellular matrix." Am J Physiol Lung Cell Mol Physiol **280**(2): L191-202.

Hackett, B. P., N. Shimizu and J. D. Gitlin (1992). "Clara cell secretory protein gene expression in bronchiolar epithelium." Am J Physiol **262**(4 Pt 1): L399-404.

Hajj, R., T. Baranek, R. Le Naour, P. Lesimple, E. Puchelle and C. Coraux (2007). "Basal cells of the human adult airway surface epithelium retain transit-amplifying cell properties." Stem Cells **25**(1): 139-148.

Harju, T., V. L. Kinnula, P. Paakko, K. Salmenkivi, J. Risteli and R. Kaarteenaho (2010). "Variability in the precursor proteins of collagen I and III in different stages of COPD." Respir Res **11**: 165.

Harkema, J. R., J. L. Mauderly, R. E. Gregory and J. A. Pickrell (1984). "A comparison of starvation and elastase models of emphysema in the rat." Am Rev Respir Dis **129**(4): 584-591.

Hayes, J. A., A. Korthy and G. L. Snider (1975). "The pathology of elastase-induced panacinar emphysema in hamsters." J Pathol **117**(1): 1-14.

Heemskerk-Gerritsen, B. A., J. H. Dijkman and A. A. Ten Have-Opbroek (1996). "Stereological methods: a new approach in the assessment of pulmonary emphysema." Microsc Res Tech **34**(6): 556-562.

Heidsiek, J. G., D. M. Hyde, C. G. Plopper and J. A. St George (1987). "Quantitative histochemistry of mucosubstance in tracheal epithelium of the macaque monkey." J Histochem Cytochem **35**(4): 435-442.

Hind, M., J. Corcoran and M. Maden (2002). "Temporal/spatial expression of retinoid binding proteins and RAR isoforms in the postnatal lung." Am J Physiol Lung Cell Mol Physiol **282**(3): L468-476.

Hind, M. and M. Maden (2004). "Retinoic acid induces alveolar regeneration in the adult mouse lung." Eur Respir J **23**(1): 20-27.

Hislop, A. (2005). "Developmental biology of the pulmonary circulation." Paediatr Respir Rev **6**(1): 35-43.

Hislop, A. A. (2002). "Airway and blood vessel interaction during lung development." J Anat **201**(4): 325-334.

Hodge, S. E. (2001). "Model-free vs. model-based linkage analysis: a false dichotomy?" Am J Med Genet **105**(1): 62-64.

Hogg, J. C. (2004). "Pathophysiology of airflow limitation in chronic obstructive pulmonary disease." Lancet **364**(9435): 709-721.

Hogg, J. C., F. Chu, S. Utokaparch, R. Woods, W. M. Elliott, L. Buzatu, R. M. Cherniack, R. M. Rogers, F. C. Sciurba, H. O. Coxson and P. D. Pare (2004). "The nature of small-airway obstruction in chronic obstructive pulmonary disease." N Engl J Med **350**(26): 2645-2653.

Holgate, S. T., J. Holloway, S. Wilson, F. Bucchieri, S. Puddicombe and D. E. Davies (2004). "Epithelial-mesenchymal communication in the pathogenesis of chronic asthma." Proc Am Thorac Soc **1**(2): 93-98.

Hong, K., E. L. Berg and R. O. Ehrhardt (2001). "Persistence of pathogenic CD4+ Th1-like cells in vivo in the absence of IL-12 but in the presence of autoantigen." J Immunol **166**(7): 4765-4772.

Hong, K. U., S. D. Reynolds, S. Watkins, E. Fuchs and B. R. Stripp (2004). "Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium." Am J Pathol **164**(2): 577-588.

Houtmeyers, E., R. Gosselink, G. Gayan-Ramirez and M. Decramer (1999). "Regulation of mucociliary clearance in health and disease." Eur Respir J **13**(5): 1177-1188.

Hovenberg, H. W., J. R. Davies and I. Carlstedt (1996). "Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells." Biochem J **318 (Pt 1)**: 319-324.

Hunninghake, G. W., J. E. Gadek, T. J. Lawley and R. G. Crystal (1981). "Mechanisms of neutrophil accumulation in the lungs of patients with idiopathic pulmonary fibrosis." J Clin Invest **68**(1): 259-269.

Ikeda, K., J. C. Clark, J. R. Shaw-White, M. T. Stahlman, C. J. Boutell and J. A. Whitsett (1995). "Gene structure and expression of human thyroid transcription factor-1 in respiratory epithelial cells." J Biol Chem **270**(14): 8108-8114.

Isakson, B. E., R. L. Lubman, G. J. Seedorf and S. Boitano (2001). "Modulation of pulmonary alveolar type II cell phenotype and communication by extracellular matrix and KGF." Am J Physiol Cell Physiol **281**(4): C1291-1299.

Ishizawa, K., H. Kubo, M. Yamada, S. Kobayashi, M. Numasaki, S. Ueda, T. Suzuki and H. Sasaki (2004). "Bone marrow-derived cells contribute to lung regeneration after elastase-induced pulmonary emphysema." FEBS Lett **556**(1-3): 249-252.

Ishizawa, K., H. Kubo, M. Yamada, S. Kobayashi, T. Suzuki, S. Mizuno, T. Nakamura and H. Sasaki (2004). "Hepatocyte growth factor induces angiogenesis in injured lungs through mobilizing endothelial progenitor cells." Biochem Biophys Res Commun **324**(1): 276-280.

Jeffery, P. K. (2001). "Remodeling in asthma and chronic obstructive lung disease." Am J Respir Crit Care Med **164**(10 Pt 2): S28-38.

Jeffery, P. K. and D. Li (1997). "Airway mucosa: secretory cells, mucus and mucin genes." Eur Respir J **10**(7): 1655-1662.

Jobe, A. H. and E. Bancalari (2001). "Bronchopulmonary dysplasia." Am J Respir Crit Care Med **163**(7): 1723-1729.

Jobe, A. H. and M. Ikegami (1998). "Surfactant and acute lung injury." Proc Assoc Am Physicians **110**(6): 489-495.

Jobe, A. H. and M. Ikegami (2001). "Prevention of bronchopulmonary dysplasia." Curr Opin Pediatr **13**(2): 124-129.

Karlinsky, J. B., R. H. Goldstein, B. Ojserkis and G. L. Snider (1986). "Lung mechanics and connective tissue levels in starvation-induced emphysema in hamsters." Am J Physiol **251**(2 Pt 2): R282-288.

Kasahara, Y., R. M. Tuder, L. Taraseviciene-Stewart, T. D. Le Cras, S. Abman, P. K. Hirth, J. Waltenberger and N. F. Voelkel (2000). "Inhibition of VEGF receptors causes lung cell apoptosis and emphysema." J Clin Invest **106**(11): 1311-1319.

Kastner, P., A. Krust, C. Mendelsohn, J. M. Garnier, A. Zelent, P. Leroy, A. Staub and P. Chambon (1990). "Murine isoforms of retinoic acid receptor gamma with specific patterns of expression." Proc Natl Acad Sci U S A **87**(7): 2700-2704.

Keil, M., G. Lungarella, E. Cavarra, P. van Even and P. A. Martorana (1996). "A scanning electron microscopic investigation of genetic emphysema in tight-skin, pallid, and beige mice, three different C57 BL/6J mutants." Lab Invest **74**(2): 353-362.

Kerr, J. S., D. J. Riley, S. Lanza-Jacoby, R. A. Berg, H. C. Spilker, S. Y. Yu and N. H. Edelman (1985). "Nutritional emphysema in the rat. Influence of protein depletion and impaired lung growth." Am Rev Respir Dis **131**(4): 644-650.

Khoor, A., M. E. Gray, W. M. Hull, J. A. Whitsett and M. T. Stahlman (1993). "Developmental expression of SP-A and SP-A mRNA in the proximal and distal respiratory epithelium in the human fetus and newborn." J Histochem Cytochem **41**(9): 1311-1319.

Khoor, A., M. T. Stahlman, M. E. Gray and J. A. Whitsett (1994). "Temporal-spatial distribution of SP-B and SP-C proteins and mRNAs in developing respiratory epithelium of human lung." J Histochem Cytochem **42**(9): 1187-1199.

Kim, K. K., M. C. Kugler, P. J. Wolters, L. Robillard, M. G. Galvez, A. N. Brumwell, D. Sheppard and H. A. Chapman (2006). "Alveolar epithelial cell mesenchymal transition develops in vivo during pulmonary fibrosis and is regulated by the extracellular matrix." Proc Natl Acad Sci U S A **103**(35): 13180-13185.

Kim, N. and T. H. Vu (2006). "Parabronchial smooth muscle cells and alveolar myofibroblasts in lung development." Birth Defects Res C Embryo Today **78**(1): 80-89.

Klann, R. C. and A. C. Marchok (1982). "Effects of retinoic acid on cell proliferation and cell differentiation in a rat tracheal epithelial cell line." Cell Tissue Kinet **15**(5): 473-482.

Knight, D. A. and S. T. Holgate (2003). "The airway epithelium: structural and functional properties in health and disease." Respirology **8**(4): 432-446.

Knudsen, L., E. R. Weibel, H. J. Gundersen, F. V. Weinstein and M. Ochs (2010). "Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach." J Appl Physiol (1985) **108**(2): 412-421.

Knudsen, L., E. R. Weibel, H. J. Gundersen, F. V. Weinstein and M. Ochs (2010). "Assessment of air space size characteristics by intercept (chord) measurement: an accurate and efficient stereological approach." J Appl Physiol **108**(2): 412-421.

Koo, J. S., A. M. Jetten, P. Belloni, J. H. Yoon, Y. D. Kim and P. Nettesheim (1999). "Role of retinoid receptors in the regulation of mucin gene expression by retinoic acid in human tracheobronchial epithelial cells." Biochem J **338 (Pt 2)**: 351-357.

Krause, G., L. Winkler, S. L. Mueller, R. F. Haseloff, J. Piontek and I. E. Blasig (2008). "Structure and function of claudins." Biochim Biophys Acta **1778**(3): 631-645.

Kresch, M. J., C. Christian, F. Wu and N. Hussain (1998). "Ontogeny of apoptosis during lung development." Pediatr Res **43**(3): 426-431.

Kuhn, C., S. Y. Yu, M. Chraplyvy, H. E. Linder and R. M. Senior (1976). "The induction of emphysema with elastase. II. Changes in connective tissue." Lab Invest **34**(4): 372-380.

Kumar, S. and G. Duester (2011). "SnapShot: retinoic acid signaling." Cell **147**(6): 1422-1422 e1421.

Kumar, V. H., S. Lakshminrusimha, M. T. El Abiad, P. R. Chess and R. M. Ryan (2005). "Growth factors in lung development." Adv Clin Chem **40**: 261-316.

Kunig, A. M., V. Balasubramaniam, N. E. Markham, D. Morgan, G. Montgomery, T. R. Grover and S. H. Abman (2005). "Recombinant human VEGF treatment enhances alveolarization after hyperoxic lung injury in neonatal rats." Am J Physiol Lung Cell Mol Physiol **289**(4): L529-535.

Kuroki, Y. and D. R. Voelker (1994). "Pulmonary surfactant proteins." J Biol Chem **269**(42): 25943-25946.

Lafuma, C., E. Frisdal, A. Harf, L. Robert and W. Hornebeck (1991). "Prevention of leucocyte elastase-induced emphysema in mice by heparin fragments." Eur Respir J **4**(8): 1004-1009.

Lancillotti, F., N. Darwiche, G. Celli and L. M. De Luca (1992). "Retinoid status and the control of keratin expression and adhesion during the histogenesis of squamous metaplasia of tracheal epithelium." Cancer Res **52**(22): 6144-6152.

Landau, D. (2006). "Epithelial paracellular proteins in health and disease." Curr Opin Nephrol Hypertens **15**(4): 425-429.

Lazzaro, D., M. Price, M. de Felice and R. Di Lauro (1991). "The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain." Development **113**(4): 1093-1104.

Lee, S. J., S. H. Kim, J. G. Kang, C. S. Kim, S. H. Ihm, M. G. Choi and H. J. Yoo (2011). "Effects of all-trans retinoic acid on sodium/iodide symporter and CCAAT/enhancer-binding protein-homologous protein under condition of endoplasmic reticulum stress in FRTL5 thyroid cells." Horm Metab Res **43**(5): 331-336.

Leikauf, G. D., M. T. Borchers, D. R. Prows and L. G. Simpson (2002). "Mucin apoprotein expression in COPD." Chest **121**(5 Suppl): 166S-182S.

Lewis, J. F. and A. H. Jobe (1993). "Surfactant and the adult respiratory distress syndrome." Am Rev Respir Dis **147**(1): 218-233.

Li, D. Y., L. K. Sorensen, B. S. Brooke, L. D. Urness, E. C. Davis, D. G. Taylor, B. B. Boak and D. P. Wendel (1999). "Defective angiogenesis in mice lacking endoglin." Science **284**(5419): 1534-1537.

Li, J. D., A. F. Dohrman, M. Gallup, S. Miyata, J. R. Gum, Y. S. Kim, J. A. Nadel, A. Prince and C. B. Basbaum (1997). "Transcriptional activation of mucin by Pseudomonas aeruginosa lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease." Proc Natl Acad Sci U S A **94**(3): 967-972.

Liebeskind, A., S. Srinivasan, D. Kaetzel and M. Bruce (2000). "Retinoic acid stimulates immature lung fibroblast growth via a PDGF-mediated autocrine mechanism." Am J Physiol Lung Cell Mol Physiol **279**(1): L81-90.

Linke, T., H. Dawson and E. H. Harrison (2005). "Isolation and characterization of a microsomal acid retinyl ester hydrolase." J Biol Chem **280**(24): 23287-23294.

Liu, C. H. (2005). "[Study on the synergic effect of all-trans retinoic acid combined with IFN-alpha on the proliferation and differentiation of HL-60 cells]." Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi **21**(5): 637-639, 642.

Lucey, E. C., R. H. Goldstein, R. Breuer, B. N. Rexer, D. E. Ong and G. L. Snider (2003). "Retinoic acid does not affect alveolar septation in adult FVB mice with elastase-induced emphysema." Respiration **70**(2): 200-205.

MacNee, W. (2001). "Oxidative stress and lung inflammation in airways disease." Eur J Pharmacol **429**(1-3): 195-207.

Madara, J. L. (1998). "Regulation of the movement of solutes across tight junctions." Annu Rev Physiol **60**: 143-159.

Maden, M. (2006). "Retinoids have differing efficacies on alveolar regeneration in a dexamethasone-treated mouse." Am J Respir Cell Mol Biol **35**(2): 260-267.

Maden, M. and M. Hind (2003). "Retinoic acid, a regeneration-inducing molecule." Dev Dyn **226**(2): 237-244.

Maeda, Y., V. Dave and J. A. Whitsett (2007). "Transcriptional control of lung morphogenesis." Physiol Rev **87**(1): 219-244.

Maestrelli, P., M. Saetta, C. E. Mapp and L. M. Fabbri (2001). "Remodeling in response to infection and injury. Airway inflammation and hypersecretion of mucus in smoking subjects with chronic obstructive pulmonary disease." Am J Respir Crit Care Med **164**(10 Pt 2): S76-80.

Mahadeva, R. and S. D. Shapiro (2002). "Chronic obstructive pulmonary disease * 3: Experimental animal models of pulmonary emphysema." Thorax **57**(10): 908-914.

Majo, J., H. Ghezzo and M. G. Cosio (2001). "Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema." Eur Respir J **17**(5): 946-953.

Makanya, A. N., R. Hlushchuk, O. Baum, N. Velinov, M. Ochs and V. Djonov (2007). "Microvascular endowment in the developing chicken embryo lung." Am J Physiol Lung Cell Mol Physiol **292**(5): L1136-1146.

Makanya, A. N., M. P. Sparrow, C. N. Warui, D. K. Mwangi and P. H. Burri (2001). "Morphological analysis of the postnatally developing marsupial lung: The quokka wallaby." Anat Rec **262**(3): 253-265.

Mao, J. T., J. G. Goldin, J. Dermand, G. Ibrahim, M. S. Brown, A. Emerick, M. F. McNitt-Gray, D. W. Gjertson, F. Estrada, D. P. Tashkin and M. D. Roth (2002). "A pilot study of all-trans-retinoic acid for the treatment of human emphysema." Am J Respir Crit Care Med **165**(5): 718-723.

Mao, J. T., D. P. Tashkin, P. N. Belloni, I. Baileyhealy, F. Baratelli and M. D. Roth (2003). "All-trans retinoic acid modulates the balance of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in patients with emphysema." Chest **124**(5): 1724-1732.

March, T. H., E. B. Barr, G. L. Finch, K. J. Nikula and J. C. Seagrave (2002). "Effects of concurrent ozone exposure on the pathogenesis of cigarette smoke-induced emphysema in B6C3F1 mice." Inhal Toxicol **14**(12): 1187-1213.

March, T. H., P. Y. Cossey, D. C. Esparza, K. J. Dix, J. D. McDonald and L. E. Bowen (2004). "Inhalation administration of all-trans-retinoic acid for treatment of elastase-induced pulmonary emphysema in Fischer 344 rats." Exp Lung Res **30**(5): 383-404.

March, T. H., F. H. Green, F. F. Hahn and K. J. Nikula (2000). "Animal models of emphysema and their relevance to studies of particle-induced disease." Inhal Toxicol **12 Suppl 4**: 155-187.

Martin, B., J. M. Bernardon, M. T. Cavey, B. Bernard, I. Carlavan, B. Charpentier, W. R. Pilgrim, B. Shroot and U. Reichert (1992). "Selective synthetic ligands for human nuclear retinoic acid receptors." Skin Pharmacol **5**(1): 57-65.

Martorana, P. A., T. Brand, C. Gardi, P. van Even, M. M. de Santi, P. Calzoni, P. Marcolongo and G. Lungarella (1993). "The pallid mouse. A model of genetic alpha 1-antitrypsin deficiency." Lab Invest **68**(2): 233-241.

Massaro, D. and G. D. Massaro (1986). "Dexamethasone accelerates postnatal alveolar wall thinning and alters wall composition." Am J Physiol **251**(2 Pt 2): R218-224.

Massaro, D. and G. D. Massaro (2001). "Pulmonary alveolus formation: critical period, retinoid regulation and plasticity." Novartis Found Symp **234**: 229-236; discussion 236-241.

Massaro, D. and G. D. Massaro (2006). "Toward therapeutic pulmonary alveolar regeneration in humans." Proc Am Thorac Soc **3**(8): 709-712.

Massaro, D., N. Teich, S. Maxwell, G. D. Massaro and P. Whitney (1985). "Postnatal development of alveoli. Regulation and evidence for a critical period in rats." J Clin Invest **76**(4): 1297-1305.

Massaro, G. D. and D. Massaro (1986). "Development of bronchiolar epithelium in rats." Am J Physiol **250**(5 Pt 2): R783-788.

Massaro, G. D. and D. Massaro (1996). "Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats." Am J Physiol **270**(2 Pt 1): L305-310.

Massaro, G. D. and D. Massaro (1997). "Retinoic acid treatment abrogates elastase-induced pulmonary emphysema in rats." Nat Med **3**(6): 675-677.

Massaro, G. D. and D. Massaro (2000). "Retinoic acid treatment partially rescues failed septation in rats and in mice." Am J Physiol Lung Cell Mol Physiol **278**(5): L955-960.

Massaro, G. D., S. Radaeva, L. B. Clerch and D. Massaro (2002). "Lung alveoli: endogenous programmed destruction and regeneration." Am J Physiol Lung Cell Mol Physiol **283**(2): L305-309.

Matter, K. and M. S. Balda (2007). "Epithelial tight junctions, gene expression and nucleo-junctional interplay." J Cell Sci **120**(Pt 9): 1505-1511.

McDowell, E. M., T. Ben, B. Coleman, S. Chang, C. Newkirk and L. M. De Luca (1987). "Effects of retinoic acid on the growth and morphology of hamster tracheal epithelial cells in primary culture." Virchows Arch B Cell Pathol Incl Mol Pathol **54**(1): 38-51.

McDowell, E. M., K. P. Keenan and M. Huang (1984). "Effects of vitamin A-deprivation on hamster tracheal epithelium. A quantitative morphologic study." Virchows Arch B Cell Pathol Incl Mol Pathol **45**(2): 197-219.

McGowan, S., S. K. Jackson, M. Jenkins-Moore, H. H. Dai, P. Chambon and J. M. Snyder (2000). "Mice bearing deletions of retinoic acid receptors demonstrate reduced lung elastin and alveolar numbers." Am J Respir Cell Mol Biol **23**(2): 162-167.

McGowan, S. E. (1992). "Extracellular matrix and the regulation of lung development and repair." FASEB J **6**(11): 2895-2904.

McGowan, S. E. (2002). "Contributions of retinoids to the generation and repair of the pulmonary alveolus." Chest **121**(5 Suppl): 206S-208S.

McGowan, S. E., C. S. Harvey and S. K. Jackson (1995). "Retinoids, retinoic acid receptors, and cytoplasmic retinoid binding proteins in perinatal rat lung fibroblasts." Am J Physiol **269**(4 Pt 1): L463-472.

McGowan, S. E. and J. S. Torday (1997). "The pulmonary lipofibroblast (lipid interstitial cell) and its contributions to alveolar development." Annu Rev Physiol **59**: 43-62.

McMenamy, K. R. and R. D. Zachman (1993). "Effect of gestational age and retinol (vitamin A) deficiency on fetal rat lung nuclear retinoic acid receptors." Pediatr Res **33**(3): 251-255.

Meshi, B., T. Z. Vitalis, D. Ionescu, W. M. Elliott, C. Liu, X. D. Wang, S. Hayashi and J. C. Hogg (2002). "Emphysematous lung destruction by cigarette smoke. The effects of latent adenoviral infection on the lung inflammatory response." Am J Respir Cell Mol Biol **26**(1): 52-57.

Mineta, K., Y. Yamamoto, Y. Yamazaki, H. Tanaka, Y. Tada, K. Saito, A. Tamura, M. Igarashi, T. Endo, K. Takeuchi and S. Tsukita (2011). "Predicted expansion of the claudin multigene family." FEBS Lett **585**(4): 606-612.

Minoo, P., H. Hamdan, D. Bu, D. Warburton, P. Stepanik and R. deLemos (1995). "TTF-1 regulates lung epithelial morphogenesis." Dev Biol **172**(2): 694-698.

Minoo, P., G. Su, H. Drum, P. Bringas and S. Kimura (1999). "Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos." Dev Biol **209**(1): 60-71.

Mitic, L. L., C. M. Van Itallie and J. M. Anderson (2000). "Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins." Am J Physiol Gastrointest Liver Physiol **279**(2): G250-254.

Moreira, A. P. and C. M. Hogaboam (2011). "Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution." J Interferon Cytokine Res **31**(6): 485-491.

Morin, P. J. (2005). "Claudin proteins in human cancer: promising new targets for diagnosis and therapy." Cancer Res **65**(21): 9603-9606.

Morishige, W. K. and N. S. Joun (1982). "Influence of glucocorticoids on postnatal lung development in the rat: possible modulation by thyroid hormone." Endocrinology **111**(5): 1587-1594.

Morrow, C. M., G. Tyagi, L. Simon, K. Carnes, K. M. Murphy, P. S. Cooke, M. C. Hofmann and R. A. Hess (2009). "Claudin 5 expression in mouse seminiferous epithelium is dependent upon the transcription factor ets variant 5 and contributes to blood-testis barrier function." Biol Reprod **81**(5): 871-879.

Nagpal, S. and R. A. Chandraratna (2000). "Recent developments in receptor-selective retinoids." Curr Pharm Des **6**(9): 919-931.

Nicod, L. P. (1999). "Pulmonary defence mechanisms." Respiration **66**(1): 2-11.

Nishi, Y., V. Boswell, T. Ansari, F. Piprawala, S. Satchi and C. P. Page (2003). "Elastase-induced changes in lung function: relationship to morphometry and effect of drugs." Pulm Pharmacol Ther **16**(4): 221-229.

Nusrat, A., J. A. Chen, C. S. Foley, T. W. Liang, J. Tom, M. Cromwell, C. Quan and R. J. Mrsny (2000). "The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction." J Biol Chem **275**(38): 29816-29822.

O'Byrne, S. M., N. Wongsiriroj, J. Libien, S. Vogel, I. J. Goldberg, W. Baehr, K. Palczewski and W. S. Blaner (2005). "Retinoid absorption and storage is impaired in mice lacking lecithin:retinol acyltransferase (LRAT)." J Biol Chem **280**(42): 35647-35657.

O'Donnell, M. D., C. M. O'Connor, M. X. FitzGerald, G. Lungarella, E. Cavarra and P. A. Martorana (1999). "Ultrastructure of lung elastin and collagen in mouse models of spontaneous emphysema." Matrix Biol **18**(4): 357-360.

Oliveira, S. S. and J. A. Morgado-Diaz (2007). "Claudins: multifunctional players in epithelial tight junctions and their role in cancer." Cell Mol Life Sci **64**(1): 17-28.

Olsen, C. O., B. E. Isakson, G. J. Seedorf, R. L. Lubman and S. Boitano (2005). "Extracellular matrix-driven alveolar epithelial cell differentiation in vitro." Exp Lung Res **31**(5): 461-482.

Paakko, P., S. Anttila, R. Sormunen, L. Ala-Kokko, R. Peura, V. J. Ferrans and L. Ryhanen (1996). "Biochemical and morphological characterization of carbon tetrachloride-induced lung fibrosis in rats." Arch Toxicol **70**(9): 540-552.

Parera, M. C., M. van Dooren, M. van Kempen, R. de Krijger, F. Grosveld, D. Tibboel and R. Rottier (2005). "Distal angiogenesis: a new concept for lung vascular morphogenesis." Am J Physiol Lung Cell Mol Physiol **288**(1): L141-149.

Pauwels, R. (2001). "Global initiative for chronic obstructive lung diseases (GOLD): time to act." Eur Respir J **18**(6): 901-902.

Pendino, F., C. Dudognon, F. Delhommeau, T. Sahraoui, M. Flexor, A. Bennaceur-Griscelli, M. Lanotte and E. Segal-Bendirdjian (2003). "Retinoic acid receptor alpha and retinoid-X receptor-specific agonists synergistically target telomerase expression and induce tumor cell death." Oncogene **22**(57): 9142-9150.

Petrache, I., V. Natarajan, L. Zhen, T. R. Medler, A. T. Richter, C. Cho, W. C. Hubbard, E. V. Berdyshev and R. M. Tuder (2005). "Ceramide upregulation causes pulmonary cell apoptosis and emphysema-like disease in mice." Nat Med **11**(5): 491-498.

Phelps, D. S. and J. Floros (1991). "Localization of pulmonary surfactant proteins using immunohistochemistry and tissue in situ hybridization." Exp Lung Res **17**(6): 985-995.

Piu, F., N. K. Gauthier, R. Olsson, E. A. Currier, B. W. Lund, G. E. Croston, U. Hacksell and M. R. Brann (2005). "Identification of novel subtype selective RAR agonists." Biochem Pharmacol **71**(1-2): 156-162.

Pryor, W. A. and K. Stone (1993). "Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite." Ann N Y Acad Sci **686**: 12-27; discussion 27-18.

Rahman, I. and W. MacNee (1996). "Role of oxidants/antioxidants in smoking-induced lung diseases." Free Radic Biol Med **21**(5): 669-681.

Rangasamy, T., C. Y. Cho, R. K. Thimmulappa, L. Zhen, S. S. Srisuma, T. W. Kensler, M. Yamamoto, I. Petrache, R. M. Tuder and S. Biswal (2004). "Genetic ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice." J Clin Invest **114**(9): 1248-1259.

Rawlins, E. L. and B. L. Hogan (2006). "Epithelial stem cells of the lung: privileged few or opportunities for many?" Development **133**(13): 2455-2465.

Rawlins, E. L. and B. L. Hogan (2008). "Ciliated epithelial cell lifespan in the mouse trachea and lung." Am J Physiol Lung Cell Mol Physiol **295**(1): L231-234.

Rawlins, E. L., T. Okubo, Y. Xue, D. M. Brass, R. L. Auten, H. Hasegawa, F. Wang and B. L. Hogan (2009). "The role of Scgb1a1+ Clara cells in the long-term maintenance and repair of lung airway, but not alveolar, epithelium." Cell Stem Cell **4**(6): 525-534.

Rawlins, E. L., L. E. Ostrowski, S. H. Randell and B. L. Hogan (2007). "Lung development and repair: contribution of the ciliated lineage." Proc Natl Acad Sci U S A **104**(2): 410-417.

Rennard, S. I. (2004). "Treatment of stable chronic obstructive pulmonary disease." Lancet **364**(9436): 791-802.

Retamales, I., W. M. Elliott, B. Meshi, H. O. Coxson, P. D. Pare, F. C. Sciurba, R. M. Rogers, S. Hayashi and J. C. Hogg (2001). "Amplification of inflammation in emphysema and its association with latent adenoviral infection." Am J Respir Crit Care Med **164**(3): 469-473.

Rogers, D. F. (2007). "Mucoactive agents for airway mucus hypersecretory diseases." Respir Care **52**(9): 1176-1193; discussion 1193-1177.

Romberger, D. J., J. D. Beckmann, L. Claassen, R. F. Ertl and S. I. Rennard (1992). "Modulation of fibronectin production of bovine bronchial epithelial cells by transforming growth factor-beta." Am J Respir Cell Mol Biol **7**(2): 149-155.

Rosas, M., B. Thomas, M. Stacey, S. Gordon and P. R. Taylor (2010). "The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B." J Leukoc Biol **88**(1): 169-180.

Roth, M. D., J. E. Connett, J. M. D'Armiento, R. F. Foronjy, P. J. Friedman, J. G. Goldin, T. A. Louis, J. T. Mao, J. R. Muindi, G. T. O'Connor, J. W. Ramsdell, A. L. Ries, S. M. Scharf, N. W. Schluger, F. C. Sciurba, M. A. Skeans, R. E. Walter, C. H. Wendt, R. A. Wise and F. S. Investigators (2006). "Feasibility of retinoids for the treatment of emphysema study." Chest **130**(5): 1334-1345.

Schneeberger, E. E. and R. D. Lynch (2004). "The tight junction: a multifunctional complex." Am J Physiol Cell Physiol **286**(6): C1213-1228.

Segel, M. J., R. Or, A. Tzurel, E. C. Lucey, R. H. Goldstein, G. Izbicki and R. Breuer (2001). "All-trans-retinoic acid (ATRA) is of no benefit in bleomycin-induced lung injury." Pulm Pharmacol Ther **14**(5): 403-407.

Seifart, C., J. P. Muyal, A. Plagens, A. O. Yildirim, K. Kohse, V. Grau, S. Sandu, C. Reinke, T. Tschernig, C. Vogelmeier and H. Fehrenbach (2011). "All-trans retinoic acid results in irregular repair of septa and fails to inhibit proinflammatory macrophages." Eur Respir J **38**(2): 425-439.

Shahzeidi, S., P. K. Aujla, T. J. Nickola, Y. Chen, M. Z. Alimam and M. C. Rose (2003). "Temporal analysis of goblet cells and mucin gene expression in murine models of allergic asthma." Exp Lung Res **29**(8): 549-565.

Shannon, J. M. (1994). "Induction of alveolar type II cell differentiation in fetal tracheal epithelium by grafted distal lung mesenchyme." Dev Biol **166**(2): 600-614.

Shannon, J. M., T. Pan, K. E. Edeen and L. D. Nielsen (1998). "Influence of the cytoskeleton on surfactant protein gene expression in cultured rat alveolar type II cells." Am J Physiol **274**(1 Pt 1): L87-96.

Shannon, J. M., T. Pan, L. D. Nielsen, K. E. Edeen and R. J. Mason (2001). "Lung fibroblasts improve differentiation of rat type II cells in primary culture." Am J Respir Cell Mol Biol **24**(3): 235-244.

Shapiro, S. D. (1995). "The pathogenesis of emphysema: the elastase:antielastase hypothesis 30 years later." Proc Assoc Am Physicians **107**(3): 346-352.

Shapiro, S. D. (2000). "Animal models for COPD." Chest **117**(5 Suppl 1): 223S-227S.

Shebani, E., S. Shahana, C. Janson, G. M. Roomans and B. H. R. group (2005). "Attachment of columnar airway epithelial cells in asthma." Tissue Cell **37**(2): 145-152.

Shenai, J. P. and F. Chytil (1994). "Effect of maternal dexamethasone treatment on fetal lung vitamin A stores in the perinatal rat." Int J Vitam Nutr Res **64**(2): 93-97.

Shiomi, T., Y. Okada, R. Foronjy, J. Schiltz, R. Jaenish, S. Krane and J. D'Armiento (2003). "Emphysematous changes are caused by degradation of type III collagen in transgenic mice expressing MMP-1." Exp Lung Res **29**(1): 1-15.

Siafakas, N. M. and E. G. Tzortzaki (2002). "Few smokers develop COPD. Why?" Respir Med **96**(8): 615-624.

Singh, G., J. Singh, S. L. Katyal, W. E. Brown, J. A. Kramps, I. L. Paradis, J. H. Dauber, T. A. Macpherson and N. Squeglia (1988). "Identification, cellular localization, isolation, and characterization of human Clara cell-specific 10 KD protein." J Histochem Cytochem **36**(1): 73-80.

Snider, G. L., E. C. Lucey and P. J. Stone (1986). "Animal models of emphysema." Am Rev Respir Dis **133**(1): 149-169.

Soini, Y. (2011). "Claudins in lung diseases." Respir Res **12**: 70.

Soini, Y., K. Kahlos, R. Sormunen, M. Saily, P. Mantymaa, P. Koistinen, P. Paakko and V. Kinnula (2005). "Activation and relocalization of caspase 3 during the apoptotic cascade of human mesothelioma cells." APMIS **113**(6): 426-435.

Soini, Y., V. Kinnula, K. Kahlos and P. Paakko (2006). "Claudins in differential diagnosis between mesothelioma and metastatic adenocarcinoma of the pleura." J Clin Pathol **59**(3): 250-254.

Sommer, A. (1983). "Effects of vitamin A deficiency on the ocular surface." Ophthalmology **90**(6): 592-600.

Srinivasan, G., E. N. Bruce, P. K. Houtz and M. C. Bruce (2002). "Dexamethasone-induced changes in lung function are not prevented by concomitant treatment with retinoic acid." Am J Physiol Lung Cell Mol Physiol **283**(2): L275-287.

Stahlman, M. T., M. E. Gray and J. A. Whitsett (1996). "Expression of thyroid transcription factor-1(TTF-1) in fetal and neonatal human lung." J Histochem Cytochem **44**(7): 673-678.

Stinchcombe, S. V. and M. Maden (2008). "Retinoic acid induced alveolar regeneration: critical differences in strain sensitivity." Am J Respir Cell Mol Biol **38**(2): 185-191.

Stripp, B. R. (2008). "Hierarchical organization of lung progenitor cells: is there an adult lung tissue stem cell?" Proc Am Thorac Soc **5**(6): 695-698.

Sun, S. Y., P. Yue, L. Mao, M. I. Dawson, B. Shroot, W. W. Lamph, R. A. Heyman, R. A. Chandraratna, K. Shudo, W. K. Hong and R. Lotan (2000). "Identification of receptor-selective retinoids that are potent inhibitors of the growth of human head and neck squamous cell carcinoma cells." Clin Cancer Res **6**(4): 1563-1573.

Swift, C. B., J. L. Hays and W. J. Petty (2008). "Distinct functions of retinoic acid receptor beta isoforms: implications for targeted therapy." Endocr Metab Immune Disord Drug Targets **8**(1): 47-50.

Synder, M., M. Drobniewski, B. Pruszczynski and M. Sibinski (2009). "Initial experience with short Metha stem implantation." Ortop Traumatol Rehabil **11**(4): 317-323.

Takubo, Y., A. Guerassimov, H. Ghezzo, A. Triantafillopoulos, J. H. Bates, J. R. Hoidal and M. G. Cosio (2002). "Alpha1-antitrypsin determines the pattern of emphysema and function in tobacco smoke-exposed mice: parallels with human disease." Am J Respir Crit Care Med **166**(12 Pt 1): 1596-1603.

Tebockhorst, S., D. Lee, A. S. Wexler and M. J. Oldham (2007). "Interaction of epithelium with mesenchyme affects global features of lung architecture: a computer model of development." J Appl Physiol **102**(1): 294-305.

Tei, M., R. Moccia and I. K. Gipson (1999). "Developmental expression of mucin genes ASGP (rMuc4) and rMuc5ac by the rat ocular surface epithelium." Invest Ophthalmol Vis Sci **40**(9): 1944-1951.

Ten Have-Opbroek, A. A. (1981). "The development of the lung in mammals: an analysis of concepts and findings." Am J Anat **162**(3): 201-219.

Tepper, J., J. Pfeiffer, M. Aldrich, D. Tumas, J. Kern, E. Hoffman, G. McLennan and D. Hyde (2000). "Can retinoic acid ameliorate the physiologic and morphologic effects of elastase instillation in the rat?" Chest **117**(5 Suppl 1): 242S-244S.

Teramoto, S., Y. Uejima, K. Teramoto, Y. Ouchi and Y. Fukuchi (1996). "Effect of age on alteration of glutathione metabolism following chronic cigarette smoke inhalation in mice." Lung **174**(2): 119-126.

Teta, M., M. M. Rankin, S. Y. Long, G. M. Stein and J. A. Kushner (2007). "Growth and regeneration of adult beta cells does not involve specialized progenitors." Dev Cell **12**(5): 817-826.

Tsao, P. N., H. Li, S. C. Wei, M. L. Ko, H. C. Chou, W. S. Hsieh and F. J. Hsieh (2004). "Expression of angiogenic factors and their receptors in postnatal mouse developing lung." J Formos Med Assoc **103**(2): 137-143.

Tschanz, S. A., B. M. Damke and P. H. Burri (1995). "Influence of postnatally administered glucocorticoids on rat lung growth." Biol Neonate **68**(4): 229-245.

Tuder, R. M., I. Petrache, J. A. Elias, N. F. Voelkel and P. M. Henson (2003). "Apoptosis and emphysema: the missing link." Am J Respir Cell Mol Biol **28**(5): 551-554.

Turner, J. and C. E. Jones (2009). "Regulation of mucin expression in respiratory diseases." Biochem Soc Trans **37**(Pt 4): 877-881.

Tzortzaki, E. G. and N. M. Siafakas (2009). "A hypothesis for the initiation of COPD." Eur Respir J **34**(2): 310-315.

Valentine, R., R. B. Rucker, C. E. Chrisp and G. L. Fisher (1983). "Morphological and biochemical features of elastase-induced emphysema in strain A/J mice." Toxicol Appl Pharmacol **68**(3): 451-461.

Van Winkle, L. S., Z. A. Johnson, S. J. Nishio, C. D. Brown and C. G. Plopper (1999). "Early events in naphthalene-induced acute Clara cell toxicity: comparison of membrane permeability and ultrastructure." Am J Respir Cell Mol Biol **21**(1): 44-53.

Vernooy, J. H., M. A. Dentener, R. J. van Suylen, W. A. Buurman and E. F. Wouters (2002). "Long-term intratracheal lipopolysaccharide exposure in mice results in chronic lung inflammation and persistent pathology." Am J Respir Cell Mol Biol **26**(1): 152-159.

Vestbo, J. (2002). "Epidemiological studies in mucus hypersecretion." Novartis Found Symp **248**: 3-12; discussion 12-19, 277-282.

Vestbo, J., E. Prescott and P. Lange (1996). "Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group." Am J Respir Crit Care Med **153**(5): 1530-1535.

Voynow, J. A., B. M. Fischer, D. E. Malarkey, L. H. Burch, T. Wong, M. Longphre, S. B. Ho and W. M. Foster (2004). "Neutrophil elastase induces mucus cell metaplasia in mouse lung." Am J Physiol Lung Cell Mol Physiol **287**(6): L1293-1302.

Wang, F., B. Daugherty, L. L. Keise, Z. Wei, J. P. Foley, R. C. Savani and M. Koval (2003). "Heterogeneity of claudin expression by alveolar epithelial cells." Am J Respir Cell Mol Biol **29**(1): 62-70.

Wang, J., K. Edeen, R. Manzer, Y. Chang, S. Wang, X. Chen, C. J. Funk, G. P. Cosgrove, X. Fang and R. J. Mason (2007). "Differentiated human alveolar epithelial cells and reversibility of their phenotype in vitro." Am J Respir Cell Mol Biol **36**(6): 661-668.

Wang, Z., T. Zheng, Z. Zhu, R. J. Homer, R. J. Riese, H. A. Chapman, Jr., S. D. Shapiro and J. A. Elias (2000). "Interferon gamma induction of pulmonary emphysema in the adult murine lung." J Exp Med **192**(11): 1587-1600.

Warburton, D. and M. K. Lee (1999). "Current concepts on lung development." Curr Opin Pediatr **11**(3): 188-192.

Warburton, D., M. Schwarz, D. Tefft, G. Flores-Delgado, K. D. Anderson and W. V. Cardoso (2000). "The molecular basis of lung morphogenesis." Mech Dev **92**(1): 55-81.

Weaver, T. E. and J. A. Whitsett (1991). "Function and regulation of expression of pulmonary surfactant-associated proteins." Biochem J **273(Pt 2)**: 249-264.

Wendel, D. P., D. G. Taylor, K. H. Albertine, M. T. Keating and D. Y. Li (2000). "Impaired distal airway development in mice lacking elastin." Am J Respir Cell Mol Biol **23**(3): 320-326.

Wert, S. E., S. W. Glasser, T. R. Korfhagen and J. A. Whitsett (1993). "Transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of transgenic mice." Dev Biol **156**(2): 426-443.

Williams, M. C. (2003). "Alveolar type I cells: molecular phenotype and development." Annu Rev Physiol **65**: 669-695.

Willis, B. C., J. M. Liebler, K. Luby-Phelps, A. G. Nicholson, E. D. Crandall, R. M. du Bois and Z. Borok (2005). "Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor-beta1: potential role in idiopathic pulmonary fibrosis." Am J Pathol **166**(5): 1321-1332.

Wilson, J. G., C. B. Roth and J. Warkany (1953). "An analysis of the syndrome of malformations induced by maternal vitamin A deficiency. Effects of restoration of vitamin A at various times during gestation." Am J Anat **92**(2): 189-217.

Winick, M. (1979). "Nutrition and aging." J Med Soc N J **76**(3): 216-217.

Wuenschell, C. W., M. E. Sunday, G. Singh, P. Minoo, H. C. Slavkin and D. Warburton (1996). "Embryonic mouse lung epithelial progenitor cells co-express immunohistochemical markers of diverse mature cell lineages." J Histochem Cytochem **44**(2): 113-123.

Yanagihara, K., M. Seki and P. W. Cheng (2001). "Lipopolysaccharide Induces Mucus Cell Metaplasia in Mouse Lung." Am J Respir Cell Mol Biol **24**(1): 66-73.

Yokohori, N., K. Aoshiba, A. Nagai and J. Respiratory Failure Research Group in (2004). "Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema." Chest **125**(2): 626-632.

Yoshimatsu, A., K. Nan-ya and I. Miki (2009). "Effect of nartograstim, a recombinant human granulocyte colony-stimulating factor on elastase-induced emphysema in rats." Arzneimittelforschung **59**(5): 248-253.

Young, R. P. and R. J. Hopkins (2011). "COPD and lung cancer linked at a molecular genetic level." Chest **140**(1): 266-267.

Zachman, R. D. (1989). "Retinol (vitamin A) and the neonate: special problems of the human premature infant." Am J Clin Nutr **50**(3): 413-424.

Zachman, R. D. and M. A. Grummer (1998). "Effect of maternal/fetal vitamin A deficiency on fetal rat lung surfactant protein expression and the response to prenatal dexamethasone." Pediatr Res **43**(2): 178-183.

Zani, A., L. Cordischi, M. Cananzi, P. De Coppi, V. V. Smith, S. Eaton and A. Pierro (2008). "Assessment of a neonatal rat model of necrotizing enterocolitis." Eur J Pediatr Surg **18**(6): 423-426.

Zhao, Y. X., L. Wang, D. Liu and R. J. Yu (2006). "[Retinoic acid inhibits tumor necrosis factor-alpha induced injury in human lung epithelial cells]." Zhonghua Yi Xue Za Zhi **86**(47): 3358-3361.

Zheng, T., Z. Zhu, Z. Wang, R. J. Homer, B. Ma, R. J. Riese, Jr., H. A. Chapman, Jr., S. D. Shapiro and J. A. Elias (2000). "Inducible targeting of IL-13 to the adult lung causes matrix metalloproteinase- and cathepsin-dependent emphysema." J Clin Invest **106**(9): 1081-1093.

Zhou, L., L. Lim, R. H. Costa and J. A. Whitsett (1996). "Thyroid transcription factor-1, hepatocyte nuclear factor-3beta, surfactant protein B, C, and Clara cell secretory protein in developing mouse lung." J Histochem Cytochem **44**(10): 1183-1193.